Co-association between Group B *Streptococcus* and *Candida albicans* promotes interactions with vaginal epithelium

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Running Head: GBS-*Candida* co-association promotes host interactions

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ABSTRACT

Group B *Streptococcus* (GBS) is a leading cause of neonatal sepsis, pneumonia and meningitis worldwide. In the majority of cases, GBS is transmitted vertically from mother to neonate, making maternal vaginal colonisation a key risk factor for neonatal disease. The fungus *Candida albicans* is an opportunistic pathogen of the female genitourinary tract, and the causative agent of vaginal thrush. Carriage of *C. albicans* has been shown to be an independent risk factor for vaginal colonisation by GBS. However, the nature of interactions between these two microbes is poorly understood. This study provides evidence of a reciprocal, synergistic interplay between GBS and *C. albicans* that may serve to promote their co-colonisation of the vaginal mucosa. GBS strains NEM316 (serotype III) and 515 (Ia) are shown to physically interact with *C. albicans*, with bacteria exhibiting tropism for candidal hyphal filaments. This interaction enhances association levels of both microbes with vaginal epithelial cell line VK2/E6E7. The ability of GBS to co-associate with *C. albicans* is dependent upon expression of hyphal-specific adhesin Als3. In turn, expression of GBS antigen I/II family adhesins (Bsp polypeptides) facilitates this co-association and confers upon surrogate *Lactococcus lactis* the capacity to exhibit enhanced interactions with *C. albicans* on vaginal epithelium. As genitourinary tract colonisation is an essential first step in the pathogenesis of GBS and *C. albicans*, the co-association mechanism reported here may have important implications for risk of disease involving both of these pathogens.

INTRODUCTION

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a leading cause of invasive disease (sepsis, pneumonia, meningitis) in neonates, and is responsible for life threatening infections in elderly and immune-compromised individuals (1-3). GBS is an opportunistic pathogen of the female genitourinary (GU) tract, with a carriage rate in Western countries of approximately 30% (2). The primary route of transmission to neonates is from the mother during or preceding birth, with transmission rates estimated at up to 50% (2). Of those neonates that are colonised, about 1% develop severe GBS disease, resulting in significant infant morbidity or mortality (2, 4).

A variety of proteins have been identified on the surface of GBS that may promote colonisation of host mucosae. These include pili (5), alpha C protein (6), BibA (7), serine-rich repeat proteins (Srr1/2) (5, 8), FbsA (9), Lmb (10), and the recently characterised antigen I/II (AgI/II) family proteins, designated BspA-D (11, 12). Many of these surface proteins have been shown to target receptors expressed directly on the cervical or vaginal epithelia, while others bind extracellular matrix (ECM) proteins such as collagen, fibrinogen, fibronectin or laminin (5, 8-10). An additional colonisation strategy for GBS, but one that remains poorly understood, is via interactions with other members of the vaginal microbiota. It is widely accepted that a ‘healthy’ vaginal microbiota is dominated (ca. 70%) by the genus *Lactobacillus*, but Gram-positive bacteria (e.g. streptococci, staphylococci), Gram-negative bacteria (e.g. *Escherichia coli*) and yeasts (e.g. *Candida albicans*) are also frequently isolated (13). Of particular relevance to GBS colonisation is a growing body of evidence indicating an association with fungus *C. albicans*. In both developed and developing countries, vaginal carriage of

C. albicans has been shown to be an independent risk factor for vaginal colonisation by GBS (14-18).

C. albicans accounts for the fourth highest rate of systemic nosocomial infection in the US (19), and as an opportunistic pathogen of the oropharynx and female GU tract is the predominant cause of both oral and vaginal thrush. Key risk factors for *C. albicans* infection are immunosuppression, use of oral contraceptives, hormone therapy, antibiotics, diabetes, and pregnancy (20). A number of colonisation determinants have been implicated in promoting candidal adhesion to and invasion of mucosae. These include proteins that are expressed on the surface of both morphological forms (blastospore and hypha) of *C. albicans* such as Als1, Eap1, Eno1, Pra1 and Tdh1 (21-25). Other major candidal adhesins, including Hwp1, Als3 and Ssa1, are exclusively expressed on the filamentous hyphae (22, 26, 27). Similarly to GBS, epithelial receptor molecules (e.g. CEACAMs, cadherins) or ECM proteins (e.g. fibronectin, laminin) have been identified as targets for these *C. albicans* adhesins (25, 27-29).

Synergistic polymicrobial interactions have already been described for *C. albicans* and a number of Gram-positive bacteria. For example, oral bacterium *Streptococcus gordonii* produces nutrient by-products that are stimulatory to *C. albicans,* enhancing the length of hyphal filaments (30). In turn, *S. gordonii* benefits from the reduced oxygen environment generated by *C. albicans* metabolism (31). Physical coadhesion between these two microbes also serves to promote retention of *C. albicans* within the oral cavity, the molecular basis of which has been identified as recognition of *C. albicans* adhesin Als3 by *S. gordonii* AgI/II family protein SspB (32). Similar interactions have been reported for *Streptococcus mutans* and *C. albicans*, for which *S. mutans*

glucosyltransferase GtfB has been shown to bind mannans on the candidal cell surface, promoting robust cross-kingdom biofilm formation within the oral cavity of rats (33). In addition to niche colonisation, interkingdom interactions may modulate disease progression. *Streptococcus oralis* and *C. albicans* synergise within the oropharynx to promote breakdown of epithelial tight junctions, resulting in enhanced systemic dissemination of *C. albicans* (34, 35). Likewise *Staphylococcus aureus* has a high affinity for binding *C. albicans* hyphae and can 'piggy back' on these filamentous forms as they infiltrate host cells to gain access to deeper tissues (36). Again, staphylococcal recognition of *C. albicans* hyphal protein Als3 is critical for this coadhesion (37).

We have recently characterised the AgI/II family polypeptide of GBS designated BspA. Alongside binding to salivary pellicle and vaginal epithelium, BspA was shown to promote coaggregation of GBS strain NEM316 with *C. albicans* under planktonic conditions (12). This study therefore aimed to build on these initial observations and determine in more detail the interkingdom interactions between GBS and *C. albicans*, and to investigate their potential to modulate the colonisation or pathogenic capabilities of these two microbes within the GU tract.

RESULTS

Planktonic interactions of GBS and *C. albicans*. The first step in exploring the interactions of GBS with *C. albicans* was to confirm their capacity to coaggregate under planktonic conditions. Two strains of GBS were tested that represent two of the most common capsular serotypes associated with neonatal disease: GBS strain 515, capsular serotype Ia, and strain NEM316, capsular serotype III (Table 1). *C. albicans*

was fluorescently-labelled with Calcofluor White, while GBS strains were labelled with FITC. Suspensions were then incubated together for 1 h before visualisation by fluorescence microscopy. Both GBS strains were able to coaggregate with *C. albicans*, indicating that these interactions are not restricted to a single capsular serotype (Fig. 1). Furthermore, as reported by (12), GBS strain NEM316 exhibited a tropism for *C. albicans* hyphae rather than blastospores. This binding pattern was also apparent with GBS strain 515, although higher levels of association were seen overall with strain NEM316 (Fig. 1). Taken together, these data confirmed that GBS can undergo planktonic interactions with *C. albicans*, but implied that levels of coaggregation may be strain-dependent.

GBS-*C. albicans* interactions with vaginal epithelial cells. Since GBS and *C. albicans* are able to coaggregate, we hypothesised that such interactions could influence the capacity of these microbes to associate with vaginal epithelium. To this end, an *in vitro* assay was developed using vaginal epithelial cell (VEC) line VK2/E6E7. In the first instance, epithelial cell monolayers were exposed either to GBS alone for 1 h, or to *C. albicans* for 1 h to initiate hyphae formation, followed by GBS for a further 1 h. Numbers of associated GBS were then enumerated by viable count (CFU) from epithelial cell lysates. While GBS strain NEM316 showed higher levels of association ($1.51x10^5$ CFU/monolayer) compared to strain 515 ($7.57x10^4$ CFU/monolayer) (Fig. 2), both strains exhibited a strong affinity for the VEC monolayers. However, significantly higher numbers of bacteria were recovered for both strains in the presence of *C. albicans*. Numbers of GBS recovered from the epithelium were 1.9-fold higher for strain NEM316 and 2.1-fold higher for strain 515 compared to their respective monospecies

samples (Fig. 2). These augmentory effects were verified by confocal microscopy, although a slightly longer incubation period was needed (5 h) to obtain bacterial cell numbers that were of sufficient abundance to be clearly visible (Fig. 3). For monospecies samples, both GBS strains were evenly distributed across the VECs, but numbers of GBS cells were higher per field of view for strain NEM316 than 515 (Fig. 3, columns 1-2). In the presence of *C. albicans*, an increase in the numbers of GBS associated with the VECs was apparent for both strains (Fig. 3, columns 3-4) compared to the monospecies equivalent. This was verified by quantification of GBS biovolume (Fig. 4A). In the presence of *C. albicans*, GBS biovolume levels were 4.6-fold and 2.8-fold higher for strains NEM316 and 515 respectively than their monospecies equivalent (Fig. 4A). Many GBS cells could be seen interacting with *C. albicans* hyphae, which formed extensive mats that overlaid the epithelial monolayers (see white arrows, Fig. 3). However, there was also a visible increase in the numbers of GBS interacting with the epithelium in areas that were not seemingly colonised by *C. albicans* (see red arrows, Fig. 3). This pattern was seen for both GBS strains. Augmentation by *C. albicans* of GBS association with VECs after 5 h was further supported by enumeration of GBS from recovered epithelial lysates, and more strikingly demonstrated the effects with GBS strain 515 (Fig. 4B).

To investigate the potential for a reciprocal relationship between GBS and *C. albicans*, the effects of GBS on *C. albicans* association with vaginal epithelium were then explored using the same *in vitro* assay. For both strains tested, the presence of GBS resulted in a 4-fold elevation in the levels of *C. albicans* recovered from the VEC monolayers compared to *C. albicans* alone (Fig. 5). These data imply that a synergistic

relationship exists between *C. albicans* and GBS, and that each microbe can enhance association of the other with vaginal epithelium.

Role of diffusible signals in GBS-*C. albicans* interactions. One potential mechanism for the enhanced recovery of both GBS and *C. albicans* when co-cultured with vaginal epithelium might be that each microbe releases some form of diffusible, chemical signal that either stimulates growth of the other or promotes its capacity to associate with VECs. To explore the first possibility, growth of GBS and *C. albicans* in single- or dual-species suspensions were compared. These studies were performed under similar conditions to the *in vitro* cell culture assay, using keratinocyte serum-free medium (K-SFM) and incubation periods of 1-2 h. No significant differences in CFU were seen for either species (Fig. 6A,B), regardless of whether grown in mono- or dual-species conditions. This implied that the presence of *C. albicans* does not affect the overall growth rate of GBS, and vice versa.

To determine if diffusible signals were modulating microbial interactions with the vaginal epithelium, GBS was incubated with VEC monolayers in K-SFM, or in spent media harvested from *C. albicans* grown in K-SFM for 1 h in the presence or absence of VECs. After 1 h incubation with VEC monolayers, numbers of associated GBS were enumerated by viable count from epithelial cell lysates. No significant differences were observed between numbers (CFU/monolayer) of GBS recovered across the different conditions (Fig. 7A).

For the reciprocal study, *C. albicans* was incubated on VEC monolayers for 1 h, and then suspensions of GBS or K-SFM alone were placed in transwell inserts above the VECs. Viable counts of *C. albicans* were determined after a further 1 h incubation.

Again, no significant differences were seen in *C. albicans* association levels with VECs in the presence or absence of GBS (Fig. 7B).

One final possibility explored was that GBS or *C. albicans* modulated the permissiveness of VECs to association with the other microbe via an active but contact-dependent mechanism. This was investigated by repeating the association assays using paraformaldehyde-fixed VECs. Fixation reduced the numbers of GBS recovered from the cell lysates overall. Nonetheless, the presence of *C. albicans* again resulted in elevated association levels of GBS (Fig. 7C) and the reciprocal effect was seen for levels of *C. albicans* recovered in the presence of GBS (Fig. 7D). Taken together, these data imply that neither intermicrobial diffusible signals nor active modulation of VEC receptor profile are required for enhanced co-association of GBS or *C. albicans* with vaginal epithelium.

Role of Bsp protein in GBS-*C. albicans* interactions. Oral streptococci have been shown to promote the colonisation and retention of *C. albicans* within the oral cavity and this is mediated, in large part, by coadhesion between the microbes (31). Having demonstrated similar coadhesion between GBS and *C. albicans*, the next step was to determine the molecular basis for this physical interaction and its contribution to the synergistic effects seen with vaginal epithelium.

We have recently shown that AgI/II family protein BspA of GBS strain NEM316 promotes coaggregation with *C. albicans* under planktonic conditions (12). We therefore wanted to build on this observation and determine if the Bsp adhesin family was important in GBS augmenting interactions of *C. albicans* with VECs. In the first instance a Δ*bspC* knockout mutant was generated in GBS strain 515, which carries only a single

copy of the *bspC* gene (a homologue of *bspA*). This strain displayed only a modest (ca. 15%) reduction in association with VECs when compared to parent strain 515 (Fig. 8A). However, it has been reported previously that streptococci can compensate for loss of antigen I/II family proteins by upregulation of alternative adhesins (38). To further explore the role of Bsp adhesins, inhibition studies were therefore performed using specific antisera. Anti-Bsp sera reduced association of wild-type GBS strains NEM316 and 515 to VECs by 46% and 63% respectively compared to preimmune control (Fig. 8B). Together these data support previous evidence that Bsp adhesins have capacity to promote GBS interactions with vaginal epithelium (12), but indicate that there are additional determinants utilised by GBS for this purpose.

In the presence of *C. albicans*, a more significant difference was seen between parent 515 and Δ*bspC* knockout strains. *C. albicans* significantly promoted recovery of both GBS strains from the epithelium compared to their respective monospecies samples. However, numbers of bacteria recovered were approximately 30% lower for mutant strain 515 Δ*bspC* than parent 515 (Fig. 8A). These data imply that BspC plays a role in mediating GBS co-association with *C. albicans*. However, additional adhesins must be involved and may compensate for the lack of BspC in strain 515 Δ*bspC*.

Given this apparent adhesin redundancy, surrogate *Lactococcus lactis* strains expressing BspA or BspC were then employed in co-association assays. This allowed the functional properties associated with the individual AgI/II family proteins to be explored in greater detail. For monospecies *L. lactis*, once again only a modest increase was seen in numbers of bacteria recovered from VECs expressing BspA or BspC compared to the empty vector control strain (Fig. 9). However, for dual species

samples, recoveries of *L. lactis* strains expressing BspA or BspC were promoted by 1.8-fold or 3-fold respectively by *C. albicans* (Fig. 9), while vector-only *L. lactis* controls were increased only slightly (<0.5-fold). Overall this implies that GBS AgI/II family proteins have capacity to promote GBS association with vaginal epithelium directly, but they likely play a greater role by promoting association via *C. albicans*.

Role of Als3 protein in GBS-*C. albicans* interactions. A possible receptor for the Bsp proteins of GBS was candidal glycoprotein Als3, since this adhesin is hypha-specific (22) and has been shown to bind the AgI/II family protein SspB of *S. gordonii* to mediate interkingdom interactions (32). A *C. albicans* strain with both alleles of the *als3* gene deleted (39), as well as a corresponding complemented strain (Δ*als3*+*als3*), were used to determine if Als3 is involved in interactions between GBS and *C. albicans*. This was first investigated under planktonic conditions, and levels of coaggregation were determined semi-quantitatively according to numbers of GBS associated with individual hyphae. Both GBS strains exhibited strong interactions with *C. albicans* wild type SC5314 and *C. albicans* Δ*als3*+*als3* strains, with the majority of hyphae recorded as binding 6-20 bacteria or >20 bacteria (Fig. 10). By contrast, the majority of *C. albicans* Δ*als3* hyphae were either devoid of bacterial cells or bound only 1-5 GBS cells (Fig. 10). Thus the expression of Als3 on candidal hyphae is required to mediate strong physical interactions with GBS under planktonic conditions.

These various *C. albicans* strains were then used to determine if Als3-mediated interactions were required to modulate GBS association with VECs. Interestingly, numbers of *C. albicans* Δ*als3* associated with VECs were not significantly different from those recovered for wild type SC5314 or *C. albicans* Δ*als3*+*als3* strains. This was

contrary to observations made by others (39) in studies with oral epithelium and implies that Als3 may exhibit tissue-specific tropism. Unlike the phenomenon observed with *C. albicans* wild type, there was no enhanced association of GBS with *C. albicans* Δ*als3* in the presence of VECs, and numbers of GBS recovered were comparable to those from monospecies samples (Fig. 11). By contrast, complementation of Δ*als3* in strain *C. albicans* Δ*als3*+*als3* restored the capacity for *C. albicans* to significantly promote GBS association with vaginal epithelium relative to GBS monospecies samples (Fig. 11).

A similar scenario was seen for the reciprocal studies to determine the role of Als3 in GBS modulation of *C. albicans* interactions with VECs. Numbers of *C. albicans* Δ*als3* cells recovered from epithelial monolayers were comparable for monospecies samples and dual-species samples incorporating either of the two GBS strains (Fig. 12). By contrast, both of the GBS strains enhanced the recovery of *C. albicans* Δ*als3*+*als3* by 2.5-fold (Fig. 12) relative to the monospecies control. These latter effects were similar to those seen previously with *C. albicans* wild type SC5314 (Fig. 5). Thus, Als3 expression by *C. albicans* is required for both GBS and *C. albicans* to modulate co-association with vaginal epithelium.

Finally, studies were performed to investigate if Bsp polypeptides of GBS can bind directly to Als3 of *C. albicans*. Again, to avoid potential issues with adhesin redundancy, surrogate expression strains were utilised. A strain of *Saccharomyces cerevisiae* had previously been generated that expresses the small allele of *C. albicans* Als3 on its cell surface (40). This *S. cerevisiae* (Als3+) strain was fluorescently-labelled with FITC, while *L. lactis* strains expressing BspA, BspC or empty vector control were labelled with TRITC. Suspensions were then incubated together for 1 h before visualisation by

fluorescence microscopy. No interactions were seen between *S. cerevisiae* (Als3+) and *L. lactis* control (Fig. 13A). By contrast, coggregation could clearly be seen with *S. cerevisiae* (Als3+) and *L. lactis* strains expressing either BspA (Fig. 13B) or BspC (Fig. 13C). Thus GBS polypeptides BspA and BspC are direct binding partners for Als3 of *C. albicans*.

DISCUSSION

Intermicrobial interactions occur at most sites of colonisation within the human body and, according to the National Institutes of Health, biofilms underpin approximately 80% of infections (41). In some instances, these interactions have antagonistic outcomes, such as those between *C. albicans* and *Pseudomonas aeruginosa*. Other partnerships are seemingly synergistic in nature, such as the interactions between *C. albicans* and *S. gordonii*, *S. oralis*, *S. mutans* or *Staph. aureus* (32, 35, 37, 42). Several studies have reported co-occurrence of GBS and *C. albicans* within the GU tract (14-18), and we recently provided evidence for coaggregation between these two microbes (12). The aims of this study were therefore to further define the interkingdom interactions of these two microbes and their capacity to modulate GU tract colonisation, an essential step in the pathogenesis of both microorganisms.

Using VEC line VK2/E6E7 as a model system, this study provides evidence that a reciprocal, synergistic relationship exists between GBS and *C. albicans* that may serve to promote their co-colonisation of the vaginal mucosa. Specifically, when incubated together, numbers of both microbes associated with the epithelial monolayers were found to be significantly higher than the numbers recovered from equivalent

295 monospecies samples. Confocal microscopy revealed extensive hyphal ‘mats’ of
296 candidal cells overlaying the epithelial monolayers to which GBS cells were attached.
297 This infers that direct physical contact (i.e. coadhesion) between GBS and *C. albicans* is
298 a key mechanism that contributes to their synergistic interplay. Thus GBS may bind
299 directly to epithelium or to adherent *C. albicans* cells, and vice versa.
300 To identify the mechanistic basis of coadhesion between GBS and *C. albicans*,
301 studies focused on the hypha-specific adhesins of *C. albicans* and specifically adhesin
302 Als3, since a distinct tropism for candidal hyphae was observed for both GBS strains
303 tested. Use of Als3 knockout and complemented strains of *C. albicans* confirmed that
304 recognition of this glycoprotein by GBS is required for effective coaggregation of these
305 two microbes under planktonic conditions, and for co-association with vaginal
306 epithelium. This correlates well with the interactions of *C. albicans* and streptococci
307 within the oral cavity reported to date (32, 34) and thus may infer that Als3 recognition
308 represents a common mechanism for *C. albicans* engagement by the *Streptococcus*
309 genus. The addition of GBS to the list of microbes that utilise Als3 as a receptor,
310 alongside other streptococci, *Staph. aureus* and *Rothia dentocariosa* (32, 37, 43), also
311 adds support to the notion that Als3 plays a major role in the capacity for *C. albicans* to
312 mediate a diverse range of polymicrobial interactions.
313 In addressing the GBS side of this synergistic partnership, this study provides
314 evidence for the role of GBS AgI/II family (Bsp) adhesins in this process. Previous work
315 has implicated BspA in facilitating coaggregation of GBS strain NEM316 with *C.*
316 *albicans* under planktonic conditions (12). These data are supported here and
317 developed to include adhesin BspC, implying that these capabilities may represent

functions that are shared across the Bsp adhesin family. Moreover, loss of BspC impaired GBS co-association with *C. albicans*, while expression of BspC by *L. lactis* enabled *C. albicans* to promote association of this surrogate host with VEC monolayers. This extends our current understanding of the properties of the adhesin family and implies that Bsp adhesins are determinants of GBS that facilitate co-association with *C. albicans* on vaginal epithelium. Moreover, coggregation between surrogate hosts expressing Als3 and Bsp adhesins adds support to the hypothesis that direct binding between Bsp polypeptides of GBS and Als3 of *C. albicans* is a mechanism that underpins, at least in part, the synergy in epithelial cell interactions between these two microbes. Interestingly, while deletion of *bspC* did not ablate co-association between GBS and *C. albicans*, deletion of both *als3* alleles effectively prevented the interaction. This indicates a role for additional GBS determinants in mediating the interkingdom relationship, and implies that these determinants may also target candidal receptor Als3. This supports the evidence that Als3 has capacity to bind multiple, diverse ligands (44).

Based on primary sequence, the AgI/II family polypeptides of GBS can be divided into four homologues: BspA and B, which share 90% sequence identity, and BspC and D, which share 99% sequence identity (12). The highest level of variation between BspA/B and BspC/D is seen within the N-terminal alanine-rich and proline-rich domains. By contrast, the V domain shares 96-100% sequence identity across all four Bsp homologues (12). The V domain has been identified as the functional region of a number of AgI/II family polypeptides (45-47), including BspA, where it was shown to promote binding of GBS NEM316 to scavenger receptor agglutinin glycoprotein-340

(12). If the V domain is also responsible for GBS co-association with *C. albicans*, the high level of sequence similarity may explain why both BspA and BspC display comparable functional properties. Delineating the precise domains within Bsp that are required for engagement with candidal Als3 will be the focus of future studies.

It is clear that direct physical contact between *C. albicans* and GBS plays a significant role in their co-association with VECs. We also considered the possibility that intermicrobial signals played a role in the processes described. However, no evidence was found for diffusible molecules released by either *C. albicans* or GBS having the capacity to significantly modulate microbial interactions with vaginal epithelium. Nonetheless, provision of additional intermicrobial binding sites may not be the only mechanism involved in the synergy with VECs. For example, in dual-species images there were patches of epithelium that were heavily colonised by GBS while seemingly devoid of *C. albicans* (Fig. 3). Fixation of VECs did not inhibit co-association between GBS and *C. albicans*, implying that these effects are not dependent upon modulation of epithelial cell biology (e.g. receptor availability). Nonetheless, it remains possible that GBS engagement with *C. albicans* alters the GBS receptor profile such that these bacteria are subsequently more permissive to interactions with VECs. The large impact of *als3* gene deletion on the GBS-*C. albicans*-VEC co-association raises the prospect that Als3 may mediate such effects. Future studies will explore these possible explanations.

To conclude, this study identifies for the first time a synergistic interplay between GBS and *C. albicans* that enhances the capacity of both microorganisms to associate with vaginal epithelial cells. Molecular determinants critical to this co-association

mechanism are identified as Bsp adhesins of GBS, and Als3 of *C. albicans*. GU tract colonisation is an essential first step in the pathogenesis of diseases such as vaginal thrush, and is a significant risk factor for GBS neonatal disease due to vertical transmission. Co-association between GBS and *C. albicans* may therefore have important implications for disease risk by both of these opportunistic pathogens. This co-association also raises the intriguing possibility of utilising a convergent immunity approach to develop novel intervention strategies, as has been explored for *C. albicans* and *Staph. aureus* (48). There is currently no vaccine against GBS disease. Furthermore, while use of intrapartum antibiotic prophylaxis (IAP) has been successful in decreasing the incidence of early-onset neonatal GBS disease in some countries, the logisitics of IAP make it an unrealistic control strategy for rural and developing countries, and IAP has had no impact on the rate of late-onset GBS infection (49, 50). The data presented here imply that better control of vaginal colonisation by *C. albicans* may restrict or reduce GBS colonisation, which in turn would reduce the risk of GBS transmission. Hence vaccines against *C. albicans*, such as the promising rAls3 vaccine that has completed phase 1 clinical trials (51), could concomitantly help to reduce the burden of neonatal GBS disease.

MATERIALS AND METHODS

Microbial strains and culture conditions. The microbial strains used in this study are listed in Table 1. GBS strains were cultured in Todd-Hewitt broth with 0.5% Yeast Extract (THY) or on THY agar plates at 37 °C, 5% CO_2. *Lactococcus lactis* was cultured in GM17 broth (M17 broth supplemented with 0.5% glucose) or on GM17 agar

plates at 30 °C in a candle jar. *Escherichia coli* was cultured aerobically in Luria Bertani (LB) broth or on LB agar plates at 37 °C. Media were supplemented with 5 µg/ml erythromycin, or with 50 µg/ml (*E. coli*) or 5 µg/ml (GBS) chloramphenicol as appropriate. Heterologous protein expression in *L. lactis* was induced from nisin-inducible plasmids by the addition of 10 ng/ml nisin. Cells from GBS and *L. lactis* broth cultures were harvested by centrifugation at 5000 *g* for 7 min.

C. albicans was cultured in YPD medium (1% Yeast Extract, 2% Mycological peptone, 2% glucose) at 37 °C with shaking (220 rpm), or maintained on Sabouraud dextrose (SAB) agar plates incubated aerobically at 37 °C. *C. albicans* cells were harvested from broth cultures by centrifugation at 5000 *g* for 5 min. *S. cerevisiae* was cultured in complete supplement medium (CSM) without uracil (ForMedium) supplemented with 0.67% yeast nitrogen base (YNB; Difco) and 2% glucose at 30 ^{o}C with shaking.

Generation of GBS knockout and *L. lactis* surrogate expression strains. A Δ*bspC* mutant was generated in GBS strain 515 by in-frame allelic replacement with a chloramphenicol resistance cassette by homologous recombination, using a method previously described (52). Briefly, a knockout construct was generated by amplifying flanking regions directly upstream and downstream of the *bspC* gene from GBS strain 515 genomic DNA using primer pairs *bspC*.F1/*bspC*.R1 and *bspC.*F2/*bspC.*R2 respectively (Table 2). A *cat* cassette was amplified from chloramphenicol-resistant plasmid pR326 using primers *cat*.F and *cat*.R (Table 2). Upstream and downstream *bspC* and *cat* amplicons were then combined by stitch PCR using primers *bspC*.F1 and *bspC*.R2. The resultant amplicon was cloned into vector pHY304 (53) via *Xba*I and

410 *Bam*HI sites, and propagated in *E. coli* Stellar^{TM} (Clontech) prior to isolation and
411 electroporation into GBS 515.
412 A *L. lactis* strain expressing BspA had been generated previously (12), and a
413 similar methodology was employed here to generate a *L. lactis* strain expressing BspC.
414 In brief, the *bspC* gene was amplified from GBS strain 515 genomic DNA using primers
415 pMSP.*bspC*.F and pMSP.*bspC*.R (Table 2). The resultant amplicon was then cloned
416 into nisin-inducible expression vector pMSP7517 (54) via *Nco*I and *Xho*I sites,
417 generating plasmid pMSP.*bspC*. This construct was transformed directly into
418 electrocompetent *L. lactis* NZ9800, as described previously (12). Transformants were
419 confirmed by plasmid isolation and PCR, while expression of BspC in *L. lactis* was
420 verified by dot immunoblot.
421 **Tissue culture.** Experiments were conducted using VK2/E6E7 cells (ATCC CRL-
422 2616), an immortalised human VEC line with a similar protein profile to the natural
423 tissue (55, 56). VECs were cultured in K-SFM (Gibco®) supplemented with 0.4 mM
424 $CaCl_2$, 0.05 mg/ml Bovine Pituitary Extract and 0.1 ng/ml Epidermal Growth Factor.
425 Upon reaching 70-80% confluence, cells were disassociated using TrypLE express
426 trypsin replacement reagent (Gibco®) before being harvested and resuspended in K-
427 SFM. Appropriate volumes of cells were seeded to fresh flasks or to assay plates, as
428 required.
429 **Visualisation of dual-species planktonic interactions.** Cells from 16 h cultures
430 of *C. albicans* were harvested, washed in YNBPT (1x Yeast Nitrogen Base, 20 mM
431 Na_2HPO_4, 0.02% tryptone, adjusted to pH 7) and suspended to OD_{600} 1.0 (equivalent to
432 10^6 cells/ml) in YNBPT. This suspension was diluted 1:10 into YNBPTG (YNBPT

433 supplemented with 0.4% glucose) and incubated at 37 °C, 220 rpm for 2 h (2 ml final
434 volume). These growth conditions have previously been shown to induce candidal
435 hyphae formation (57).
436 GBS cells were harvested from 16 h cultures, washed in YNBPT, and then
437 suspended in 1.5 mM fluorescein isothiocyanate (FITC) dissolved in carbonate buffer
438 (100 mM NaCl, 50 mM Na_2CO_3), and incubated for 30 min with gentle agitation. GBS
439 cells were harvested, washed three times in carbonate buffer and the pellet suspended
440 and adjusted to OD_{600} 0.5 (equivalent to $5x10^7$ cells/ml) in YNBPTG. GBS suspension
441 (1 ml) was added to that of *C. albicans* and incubated at 37 °C for a further 1 h with
442 shaking. Calcofluor White (0.00001% in dH_2O) was added before visualisation of 10 µl
443 samples by fluorescence microscopy.
444 For quantification assays, approximately 40 images were taken of randomly
445 selected hyphae from each experimental group. Interactions were scored into one of
446 four groups, similar to the method reported previously (32): 0 interacting bacteria per
447 hypha, 1-5 bacteria, 6-20 bacteria, and more than 20 bacteria per hypha.
448 In a variation of this assay, *Saccharomyces cerevisiae* cells were harvested from a
449 16 h overnight broth culture in CSM broth, washed once in YNBPT (5 ml), and stained
450 in 1.5 mM FITC for 30 min with gentle agitation. *S. cerevisiae* were harvested and
451 washed three times in carbonate buffer. The pellet was suspended and adjusted to
452 OD_{600} 1.0 (equivalent to 10^6 cells/ml) in YNBPTG, before 1:5 dilution into YNBPTG (final
453 volume 2 ml). This suspension was incubated at 30 °C, 220 rpm for 3 h. *L. lactis* strains
454 were harvested from a 16 h overnight broth culture and washed once in YNBPT before
455 suspension in 2 mL TRITC (0.1 mg/ml in carbonate buffer) and incubated for 30 min

456 with gentle agitation. *L. lactis* cells were harvested, washed three times in carbonate
457 buffer and adjusted to OD_{600} 0.5 in YNBPTG (equivalent to $5x10^7$ cells/ml). Adjusted *L.*
458 *lactis* suspension (1 ml) was added to *S. cerevisiae* and incubated for a further 1 h at 30
459 °C, 220 rpm, before visualisation of 10 µl samples by fluorescence microscopy.
460 **Microbial growth in dual-species broth cultures.** Cells from overnight (16 h) *C.*
461 *albicans* suspension culture were harvested and washed once in phosphate-buffered
462 saline (PBS). The pellet was suspended and adjusted to OD_{600} 1.0 in K-SFM before a
463 1:10 dilution into K-SFM (2 ml final volume) and incubation at 37 °C, 220 rpm for 2 h.
464 Cells from overnight GBS broth cultures were harvested, washed once in PBS, and
465 suspended in K-SFM at OD_{600} 0.5. GBS suspension (1 ml) was added to *C. albicans*
466 suspension and the mixture incubated at 37 °C for a further 1 h. Planktonic suspensions
467 were vortex mixed for 15 s before serial 10-fold dilutions into THY broth. Numbers of
468 microorganisms were detected by viable count (CFU) on either THY agar plates (GBS)
469 supplemented with 50 µg/ml nystatin to inhibit *C. albicans* growth, or SAB agar plates
470 (*C. albicans*) supplemented with 5 µg/ml erythromycin to inhibit GBS growth.
471 **Epithelial association assay.** Epithelial association assays were conducted as
472 described by (5) with a few modifications. VECs were seeded into a 24-well plate at
473 $2x10^5$ cells/well and incubated until confluent at 37 °C, 5% CO_2 (48-72 h). *C. albicans*
474 cells were diluted in K-SFM to obtain approximately $5x10^5$ cells/ml, while GBS or *L.*
475 *lactis* cells were diluted into K-SFM to obtain approximately $5x10^5$ cells/ml.
476 Wells containing VEC monolayers were washed once with PBS and
477 approximately $5x10^5$ bacteria or *C. albicans* (1 ml; MOI 2.5) were then added to each
478 well. Bacterial suspensions were incubated at 37 °C, 5% CO_2 for 1 h, while *C. albicans*

suspensions were incubated for 2 h. For dual species assays, *C. albicans* suspensions were incubated for 1 h before the medium was replaced by GBS or *L. lactis* and incubated for a further 1 h. For all assays, wells were then washed three times with PBS before incubation for 15 min with TrypLE™, followed by two ice cold water incubations, lasting 20 min each, to lyse the VECs. Lysates were serially diluted onto THY (GBS), GM17 (*L. lactis*) or SAB (C*. albicans*) agar plates and viable counts determined, as described above. It was confirmed both visually and by monitoring levels of lactate dehydrogenase (LDH) released into the culture supernatants that epithelial monolayers remained intact and viable over the time periods for mono- or dual-species association assays.

In a variation of this assay, VEC monolayers were fixed in 2% paraformaldehyde overnight prior to incubation with cell suspensions of *C. albicans* and/or GBS. Alternatively, GBS suspensions were prepared as described above and preincubated at room temperature with 10 µg/ml rabbit preimmune or anti-Bsp sera (Eurogentec) for 30 min, prior to incubation at 37 ^{o}C for 1 h with VEC monolayers.

Spent media studies. VECs were seeded in a 24-well plate and grown to confluence. *C. albicans* was prepared as described above, and incubated with the VECs, or grown planktonically in K-SFM medium, for 1 h. *C. albicans* media were then collected and sterilised by filtration through a 0.2 µm filter. GBS suspensions, prepared as above, were adjusted to OD_{600} 1.0 in K-SFM, before a 1:200 dilution into either fresh K-SFM, K-SFM from *C. albicans* planktonic growth, or K-SFM from *C. albicans* growth on VK2/E6E7 monolayers. Aliquots (1 ml) were added to VEC monolayers and

501 incubated for 1 h. VECs were disassociated and lysed as above, with CFU of GBS
502 determined by serial dilution and viable count on THY agar plates.
503 **Transwell studies.** VECs were seeded in a 24-well plate and grown to
504 confluence. *C. albicans* cells were prepared as above, and incubated with VEC
505 monolayers for 1 h before the medium was replaced with 1 ml K-SFM. Transwell inserts
506 with high density pores of 0.4 µm (Sarstedt) were placed into wells. GBS suspensions in
507 K-SFM (OD_{600} 1.0) were diluted 1:100 into K-SFM. Aliquots (0.5 ml) were added to the
508 transwell inserts and the plates incubated for a further 1 h. The inserts were removed,
509 remaining VECs were disassociated and lysed as described above, and *C. albicans*
510 CFU determined by serial dilution and viable count on SAB agar plates.
511 **Confocal microscopy.** For visualisation by confocal microscopy, VEC
512 monolayers were grown on 19 mm glass cover slips in a 12-well plate until confluent.
513 The epithelial association assay was then carried out as described above, except that
514 the time was extended by 4 h. Calcofluor White (1 µl) was added to stain the chitin in
515 the *C. albicans* cell wall, and the cover slips then fixed in 2% paraformaldehyde. Triton
516 X-100 (0.3%) was used to permeabilise the epithelial cells before blocking in 2% BSA.
517 Bacteria were stained with a mouse anti-GBS antibody (1.B.501, Santa Cruz
518 Biotechnology) followed by a goat anti-mouse Alexafluor-488 conjugated antibody
519 (Fisher), both of which were used at a dilution of 1:200. The F-actin of the epithelial cells
520 was stained with phalloidin-TRITC (Sigma). Cover slips were then mounted onto glass
521 slides using Vectashield (Vector Labs), and imaged on a Leica SP5-AOBS confocal
522 laser scanning microscope (CSLM) attached to a Leica DM I6000 inverted
523 epifluorescence microscope. Images were processed using Volocity® software and

524 Imaris® v7.5 software (Bitplane AG, Zurich, Switzerland) was used to calculate
525 biovolumes (μm^3).
526 **Statistical analyses.** All assays were performed in triplicate unless otherwise
527 stated. Data were analysed using unpaired Student's *t* tests with Bonferroni correction,
528 as appropriate.
529
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537
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FIGURE LEGENDS

FIG 1 Fluorescence micrographs of planktonic interactions between *C. albicans* and GBS. *C. albicans* SC5314 was grown in YNBPTG for 2 h at 37 °C, 220 rpm before addition of (A) GBS strain NEM316 or (B) GBS strain 515, and incubation for a further 1 h. GBS was labelled with FITC (green), while *C. albicans* was labelled with Calcofluor White (blue). Scale bars, 20 μm.

FIG 2 Effects of *C. albicans* SC5314 on association of GBS with VECs. VEC monolayers were incubated with GBS suspensions (MOI 2.5) for 1 h (open bars) or with *C. albicans* (MOI 2.5) for 1 h followed by GBS for a further 1 h (black bars). Monolayers were then lysed and numbers of associated GBS enumerated by serial dilution onto

THY agar supplemented with 50 µg/mL nystatin. * indicates $P<0.05$ compared to monospecies controls, as determined by unpaired Student's *t*-test; n = 4.

FIG 3 Representative confocal micrographs of *C. albicans*-GBS association with VECs. VEC monolayers were incubated with GBS alone for 5 h (columns 1 and 2) or with *C. albicans* for 1 h followed by GBS for a further 5 h (columns 3 and 4). Cells were then fixed, stained and mounted onto glass slides. GBS was labelled using Alexafluor-488-conjugated antibody (green), while *C. albicans* was labelled with Calcofluor White (blue), and VECs were labelled with phalloidin-TRITC (red). GBS strains NEM316 (top panels) and 515 (bottom panels) were tested. Columns 2 and 4 are duplicates of columns 1 and 3, respectively, in which the red filter (i.e. the VECs) has been removed (VK2/E6E7 off). Scale bars, 100 µm. White arrows indicate areas where GBS binds *C. albicans* hyphae, while red arrows indicate areas where GBS is found in the absence of *C. albicans*.

FIG 4 Effects of *C. albicans* on association of GBS with VECs following 5 h incubation. (A) Quantification of GBS from confocal micrographs illustrated in Fig. 3. Images were processed using Volocity® software and Imaris® software was used to calculate GBS biovolumes (μm^3). (B) Quantification of GBS by viable count. VEC monolayers were incubated with GBS suspensions for 5 h (open bars) or with *C. albicans* for 1 h followed by GBS for a further 5 h (black bars). Monolayers were then lysed and numbers of associated GBS enumerated by serial dilution onto THY agar supplemented with 50

μg/mL nystatin. ** indicates *P*<0.01 compared to monospecies controls, as determined by unpaired Student's *t*-test; n = 4.

FIG 5 Effects of GBS on association of *C. albicans* SC5314 (WT) with VECs. VEC monolayers were incubated with *C. albicans* cells for 1 h to allow production of hyphae. GBS suspensions were then added for a further 1 h, before monolayers were lysed. Numbers of associated *C. albicans* were enumerated by serial dilution onto SAB agar supplemented with 5 μg/mL erythromycin. * indicates *P*<0.05 compared to the monospecies control, as determined by unpaired Student's *t*-test with Bonferroni correction; n = 3.

FIG 6 Growth of *C. albicans* or GBS in mono- or dual-species suspension culture. K-SFM broth cultures were inoculated with *C. albicans* SC5314 (WT) at 37 °C, 220 rpm for 1 h before addition of GBS and incubation for a further 1 h (black bars). Alternatively broth cultures were inoculated with *C. albicans* or GBS alone and incubated for 2 h or 1 h respectively (open bars). *C. albicans* CFU/mL were then determined by viable count onto SAB agar supplemented with 5 μg/mL erythromycin (A), while GBS CFU/mL were determined by viable count onto THY agar supplemented with 50 μg/mL nystatin (B). 'NS' indicates *P*>0.05 compared to the monospecies control, as determined by unpaired Student's *t*-test; n = 3.

FIG 7 Role of contact-independent mechanisms or fixation in modulating interactions of *C. albicans* or GBS with VECs. (A) *C. albicans* SC5314 (WT) was grown on VEC

773 monolayers, or planktonically in K-SFM medium, for 1 h before spent media were
774 collected and filter sterilised. GBS cells were incubated in these spent media on VECs
775 for 1 h, before monolayers were lysed and numbers of associated GBS enumerated by
776 serial dilution onto THY agar. 'NS' indicates *P*>0.05 compared to the blank K-SFM
777 control, as determined by unpaired Student's *t*-test; n = 3. (B) *C. albicans* was grown on
778 VEC monolayers for 1 h, before GBS suspensions or K-SFM alone were placed into
779 transwell baskets suspended above. After a further 1 h incubation, *C. albicans* was
780 enumerated by serial dilution onto SAB agar. (C,D) VEC monolayers were fixed with 2%
781 paraformaldehyde and then incubated with GBS suspensions (MOI 2.5) for 1 h (open
782 bars) or with *C. albicans* (MOI 2.5) for 1 h followed by GBS for a further 1 h (black bars).
783 Monolayers were then lysed and GBS CFU/mL determined by viable count onto THY
784 agar supplemented with 50 μg/mL nystatin (C), while *C. albicans* CFU/mL were
785 determined by viable count onto SAB agar supplemented with 5 μg/mL erythromycin
786 (D). 'NS' indicates *P*>0.05, ** indicates *P*<0.01 compared to monospecies controls, as
787 determined by unpaired Student's *t*-test with Bonferroni correction; n = 4 (A,B) or 3
788 (C,D).
789
790 **FIG 8** Effects of *C. albicans* or Bsp antisera on the association of GBS wild type or
791 isogenic mutant strains with VECs. (A) VEC monolayers were incubated with GBS wild
792 type (WT) 515 or mutant Δ*bspC* cell suspensions (MOI 2.5) for 1 h (open bars), or with
793 *C. albicans* SC5314 (MOI 2.5) for 1 h followed by addition of strain 515 suspensions for
794 a further 1 h (black bars). Monolayers were then lysed and numbers of associated GBS
795 were enumerated by serial dilution onto THY agar supplemented with 50 μg/mL

nystatin. ** indicates significance relative to monospecies controls; Ω indicates significance relative to wild type monospecies control; § indicates significance relative to wild type in the presence of *C. albicans*. (B) GBS cell suspensions were preincubated with preimmune (open bars) or anti-Bsp (black bars) sera, before incubation with VEC monolayers (MOI 2.5) for 1 h and enumeration from cell lysates by viable count. ** indicates significance relative to preimmune controls. Significance indicates $P<0.01$, as determined by unpaired Student's *t*-test with Bonferroni correction; n = 3.

FIG 9 Effects of *C. albicans* on the association of *L. lactis* Bsp surrogate expression strains with VECs. VEC monolayers were incubated with suspensions of *L. lactis* pMSP vector control, pMSP.BspA or pMSP.BspC (MOI 2.5) for 1 h (open bars), or with *C. albicans* SC5314 (MOI 2.5) for 1 h followed by addition of *L. lactis* suspensions for a further 1 h (black bars). Monolayers were then lysed and numbers of associated *L. lactis* were enumerated by serial dilution onto GM17 agar supplemented with 50 μg/mL nystatin. ** indicates significance relative to monospecies controls; Ω indicates significance relative to pMSP empty vector control; § indicates significance relative to pMSP empty vector control in the presence of *C. albicans*. Significance indicates $P<0.01$, as determined by unpaired Student's *t*-test with Bonferroni correction; n = 4.

FIG 10 Role of Als3 in planktonic interactions between *C. albicans* and GBS. (A) Fluorescence micrographs of planktonic interactions between *C. albicans* Δ*als3* (left panel) or Δ*als3*+*als3* complemented strain (right panel) with GBS strains NEM316 (top panels) or 515 (bottom panels). *C. albicans* was grown in YNBPTG for 2 h at 37 °C, 220

rpm before addition of GBS and incubation for a further 1 h. GBS was labelled with FITC (green), while *C. albicans* was labelled with Calcofluor White (blue). Scale bars, 20 μm. Note, interactions of GBS strains with *C. albicans* SC5314 wild type are shown in Fig. 1. (B) Semi-quantitation of numbers of *C. albicans* hyphae with 0, 1-5, 6-20 or >20 interacting GBS, based on approximately 40 randomly selected images from each experimental group. * indicates $P<0.05$; 'NS' indicates $P>0.05$, as determined by linear regression analysis of datasets; n = 4.

FIG 11 Role of Als3 in synergistic effects of *C. albicans* on association of GBS with VECs. VEC monolayers were incubated with GBS suspensions for 1 h (open bars) or with *C. albicans* SC5314 wild type (WT) (black bars), Δ*als3* (grey bars) or Δ*als3*+*als3* (hashed bars) for 1 h, followed by GBS for a further 1 h. Monolayers were lysed and then numbers of associated GBS were enumerated by serial dilution onto THY agar supplemented with 50 μg/mL nystatin. ** indicates $P<0.01$; 'NS' indicates $P>0.05$, as determined by unpaired Student's *t*-test with Bonferroni correction; n = 4.

FIG 12 Role of Als3 in synergistic effects of GBS on association of *C. albicans* with VECs. VEC monolayers were incubated with *C. albicans* SC5314 wild type (WT) (open bars), Δ*als3* (black bars) or Δ*als3*+*als3* (striped bars) for 1 h followed by GBS for a further 1 h. Monolayers were lysed and numbers of associated *C. albicans* were enumerated by serial dilution onto SAB agar supplemented with 5 μg/mL erythromycin. ** indicates $P<0.01$; 'NS' indicates $P>0.05$, as determined by unpaired Student's *t*-test with Bonferroni correction; n = 4.

842

843 **FIG 13** Fluorescence micrographs of planktonic interactions between *S. cerevisiae* Als3
844 and *L. lactis* Bsp surrogate expression strains. *S. cerevisiae* (Als3+) was grown in
845 YNBPTG for 3 h at 30 °C, 220 rpm before addition of (A) *L. lactis* (pMSP control), (B) *L.*
846 *lactis* (pMSP.BspA) or (C) *L. lactis* (pMSP.BspC) and incubation for a further 1 h. *L.*
847 *lactis* was labelled with TRITC (red), while *S. cerevisiae* was labelled with FITC (green).
848 Scale bars, 20 μm.

TABLE 1 Microbial strains used in this study

Strain or plasmid	Unique ID #	Relevant genotype	Reference or source
C. albicans			
SC5314	UB1843	Wild type	Neil Gow, Univ Aberdeen
	UB1941	Δ*als3*	(39)
	UB1940	Δ*als3*+pUL.*als3*	(39)
S. cerevisiae			
BY4742	UB2156	pBC542-*ALS3sm*	(40)
S. agalactiae			
NEM316	UB1931	Wild type	Shaynoor Dramsi, Institut Pasteur
515	UB2410	Wild type	Victor Nizet, Univ California San Diego
515	UB2873	Δ*bspC*	This study
L. lactis			
NZ9800	UB2635	pMSP	(12)
	UB2658	pMSP.*bspA*	(12)
	UB2659	pMSP.*bspC*	This study
Plasmids			
pMSP7517		*E.coli*-enterococcal shuttle vector	(54)

		containing *E. faecalis* *prgB* under control of *nisA* promoter; erythromycin^{R}	
	pMSP.*bspC*	pMSP7517-derived containing *bspC* from GBS 515 in place of *prgB*	
	pR326	chloramphenicol^{R}	(58)

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851

852

853

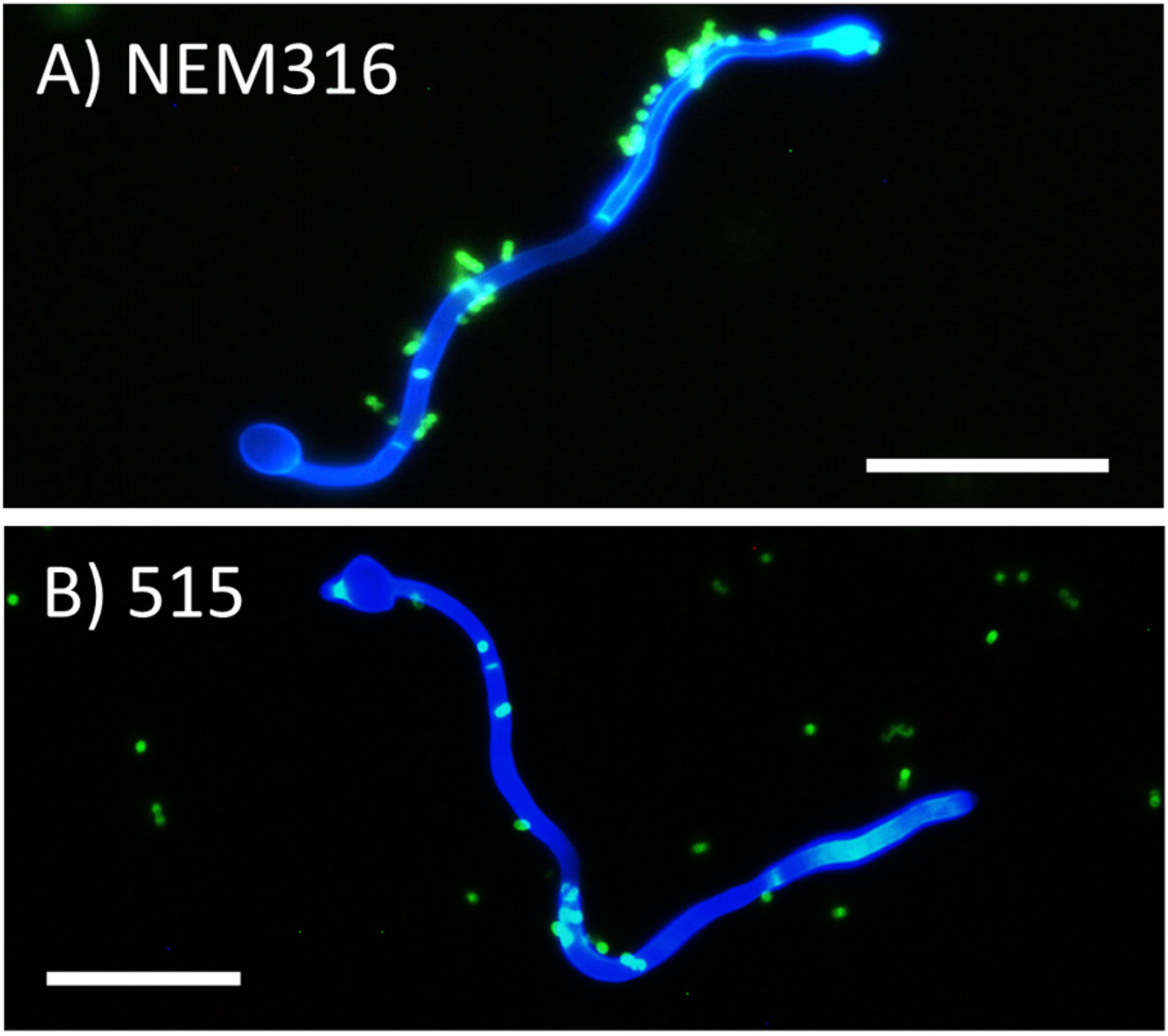
854 **TABLE 2** Primers used in this study

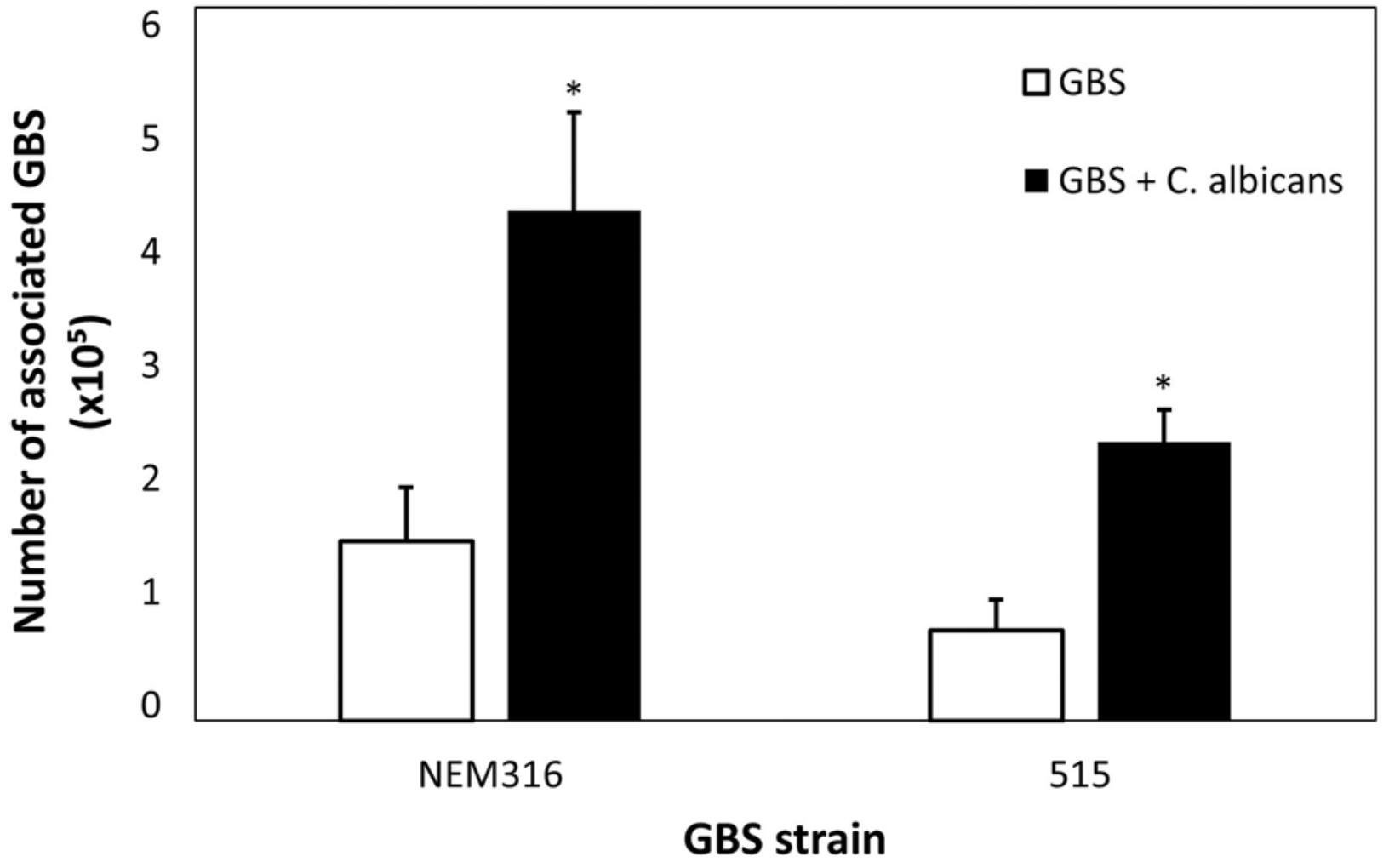
Primer name	Sequence[a]
bspC.F1	GCTCTAGAGCAATTAGCAGATGCACAG
bspC.R1	TAAAATCAAAGGAGAAAATATGAACTTTA
*bspC.*F2	GCTTTTATAATCAATATTCAGAAGCACTTG
*bspC.*R2	CGGGATCCGAGCCAAATTACCCCTCC
cat.F	AGAAAATATGAACTTTAATAAAATTGATTTAG
cat.R	TGAATATTGATTATAAAAGCCAGTCATTAGG
pMSP.*bspC*.F	CATGCCATGGAGGAGGAAATATGTATAAAAATCAAAAC
pMSP.*bspC*.R	CCGCTCGAGGCAGGTCCAGCTTCAAATC

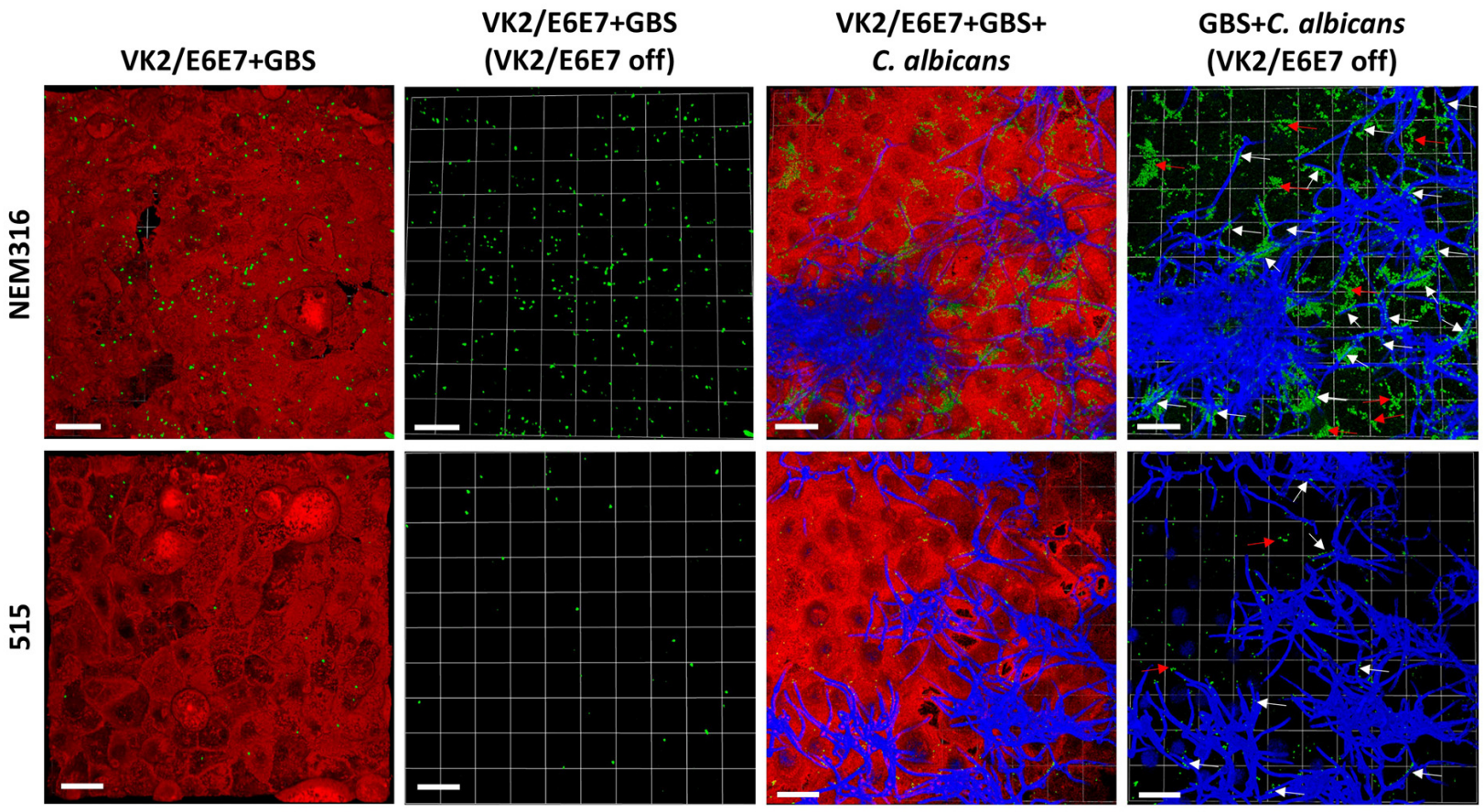
855

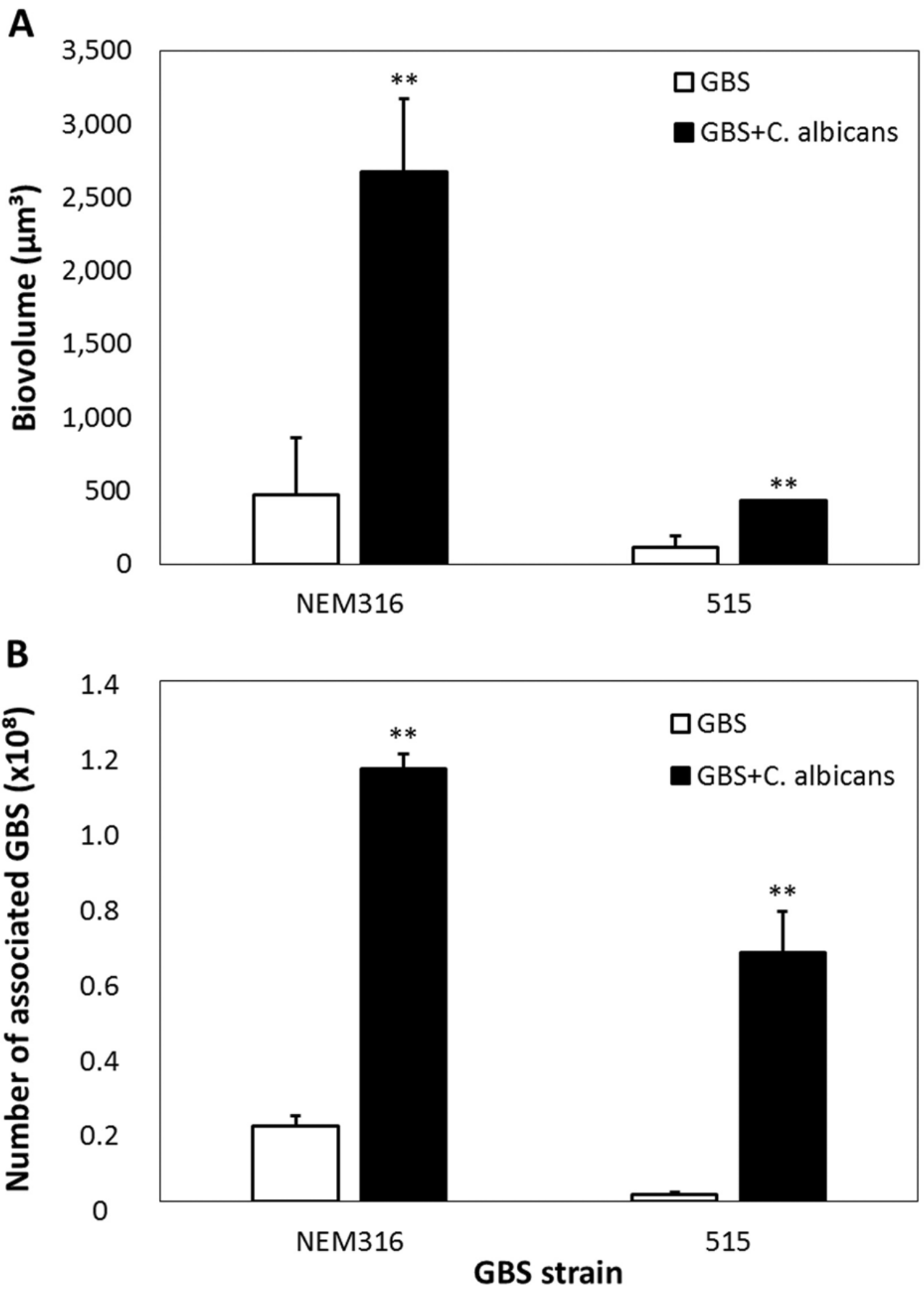
856 [a] Restriction endonuclease sites are underlined.

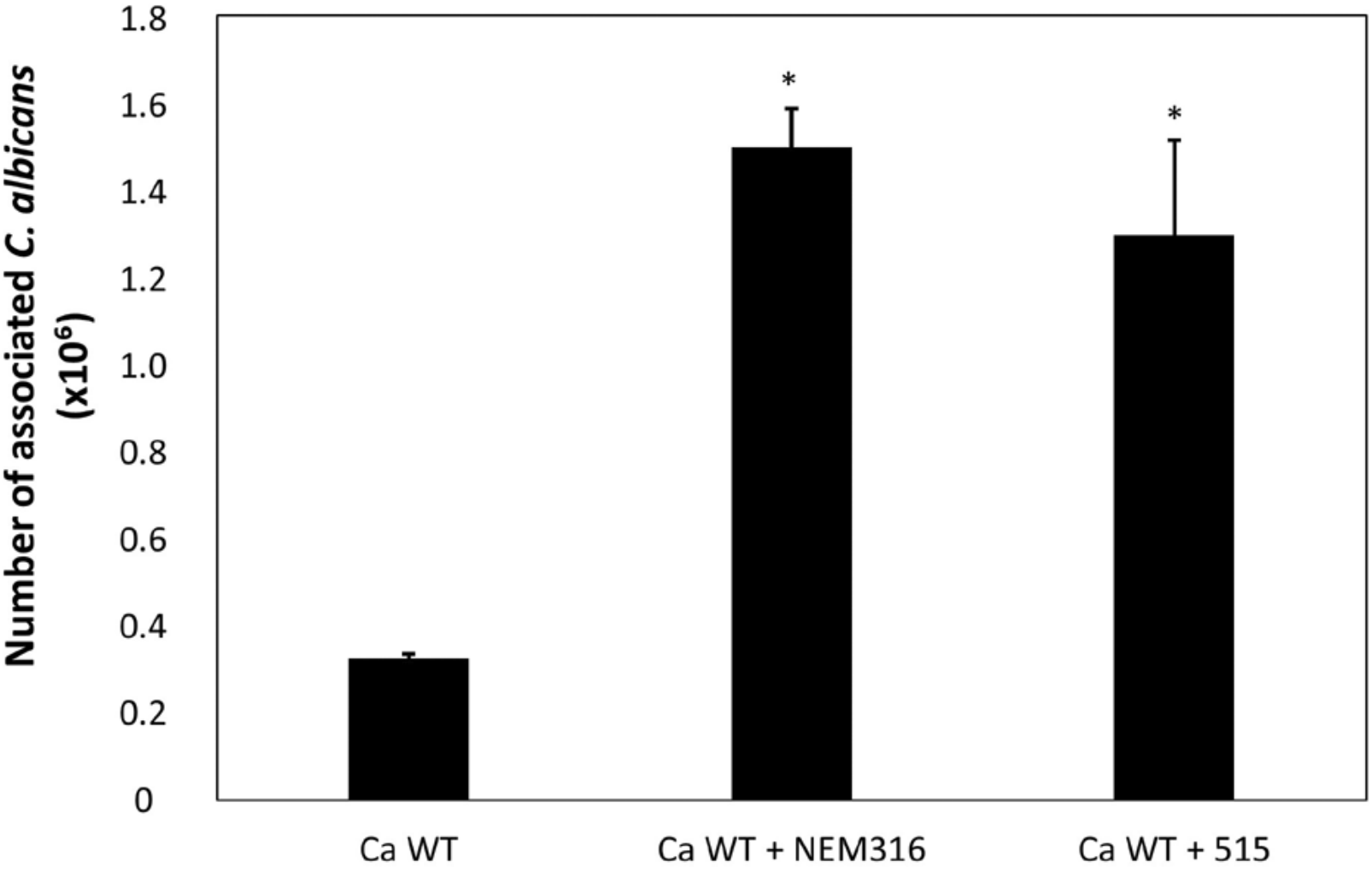
857

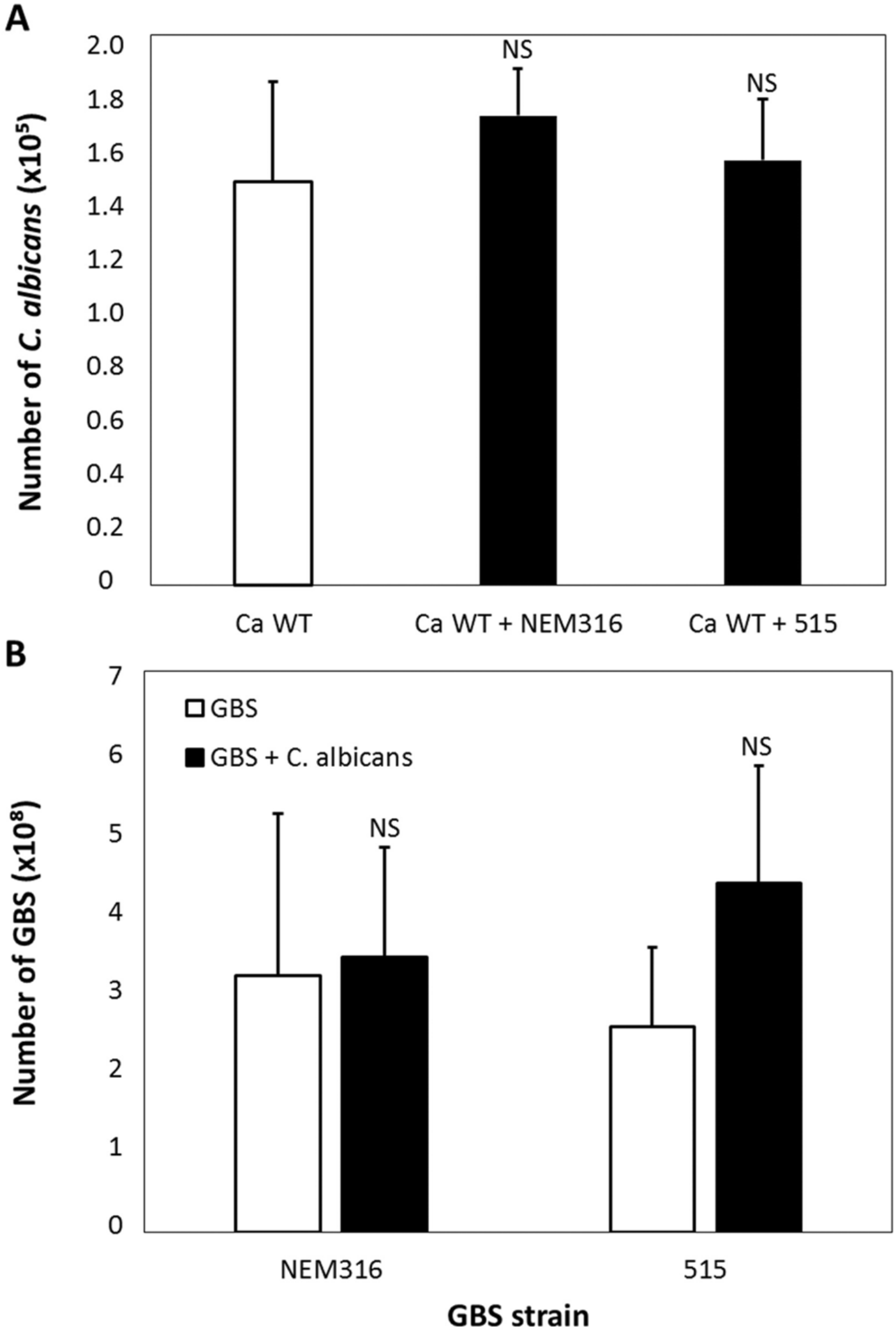

A) NEM316
B) 515

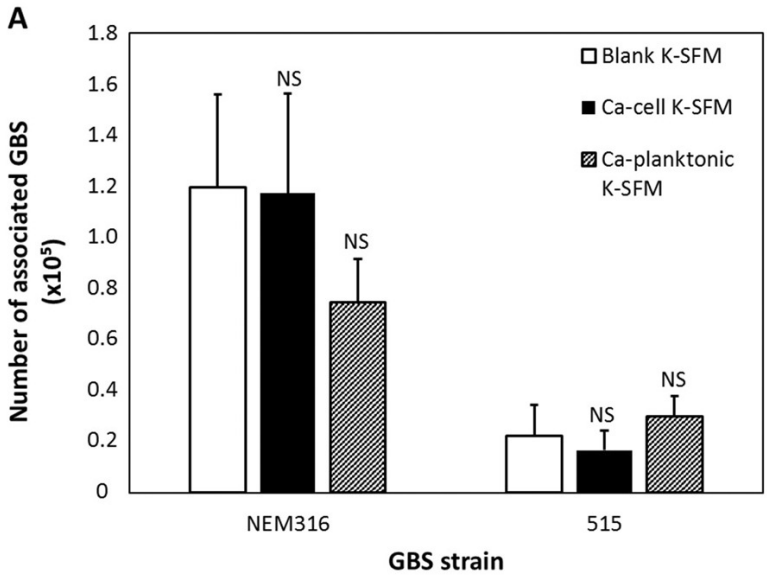

Number of associated GBS (x10⁵)
6
5
4
3
2
1
0
GBS
GBS + C. albicans
*
*
NEM316
515
GBS strain

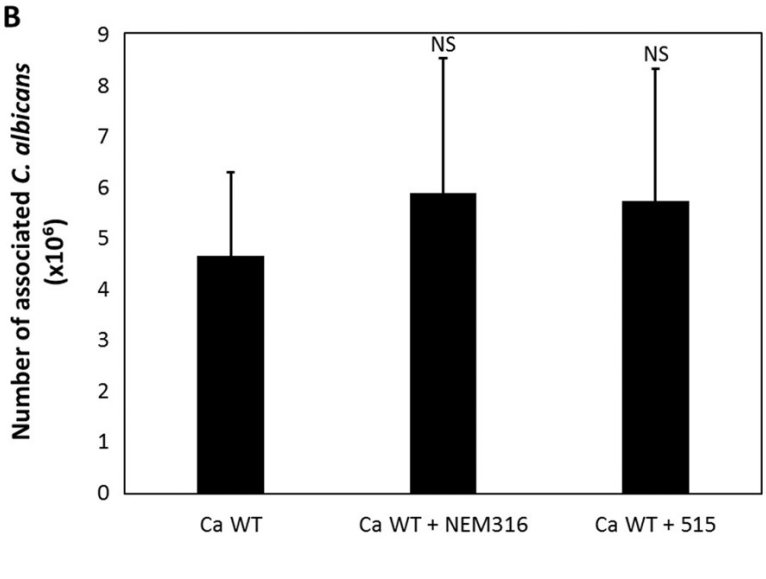

VK2/E6E7+GBS
VK2/E6E7+GBS
(VK2/E6E7 off)
VK2/E6E7+GBS+
C. albicans
GBS+C. albicans
(VK2/E6E7 off)
NEM316
515

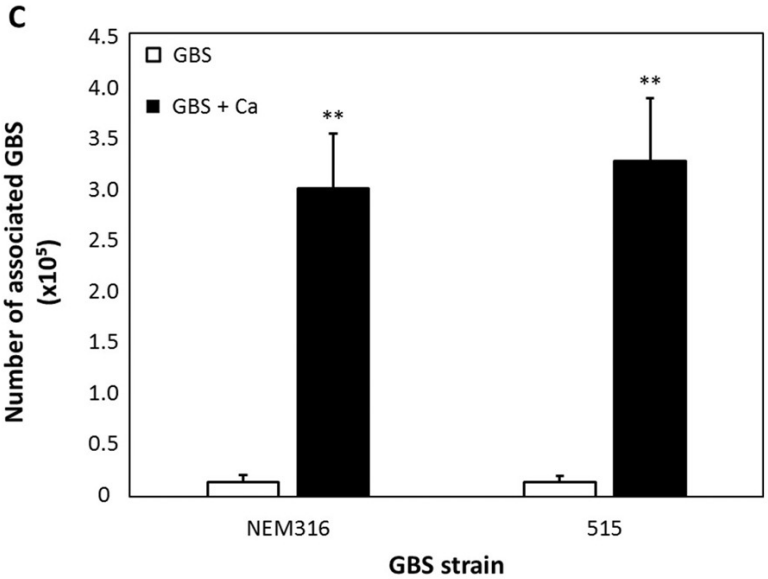

A
3,500
3,000
2,500
2,000
1,500
1,000
500
0
Biovolume (µm³)
**
**
GBS
GBS+C. albicans
NEM316
515
B
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Number of associated GBS (x10⁸)
**
**
GBS
GBS+C. albicans
NEM316
515
GBS strain

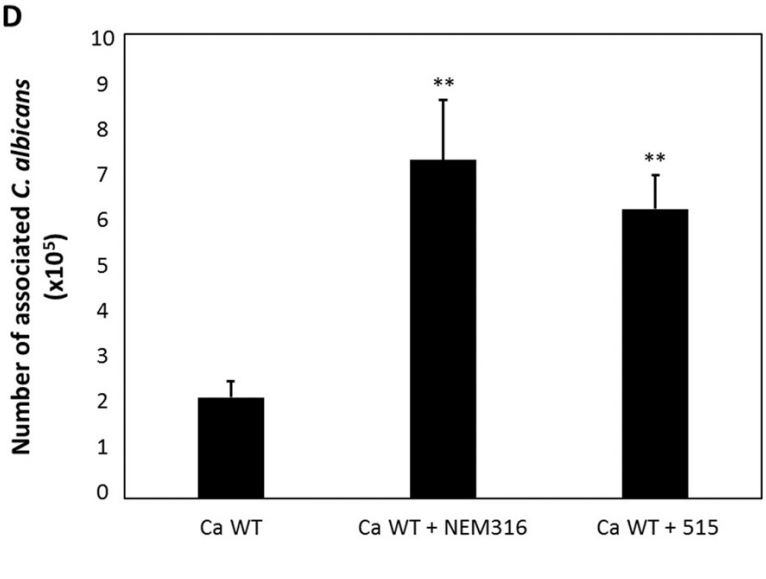

Number of associated C. albicans (x10^6)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
Ca WT
Ca WT + NEM316
Ca WT + 515

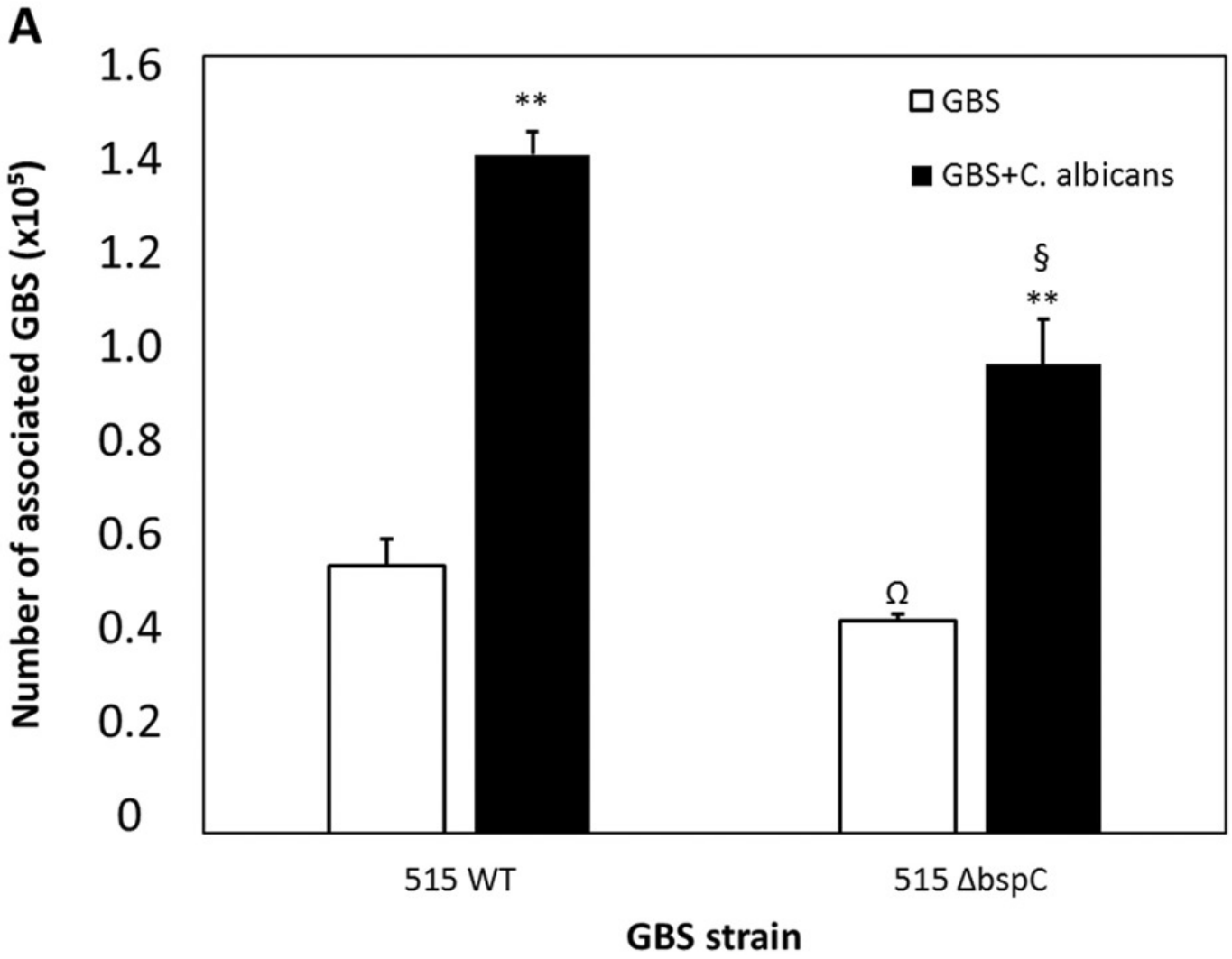

A
Number of C. albicans (x10⁵)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
NS
NS
Ca WT
Ca WT + NEM316
Ca WT + 515
B
Number of GBS (x10⁸)
7
6
5
4
3
2
1
0
GBS
GBS + C. albicans
NS
NS
NEM316
515
GBS strain

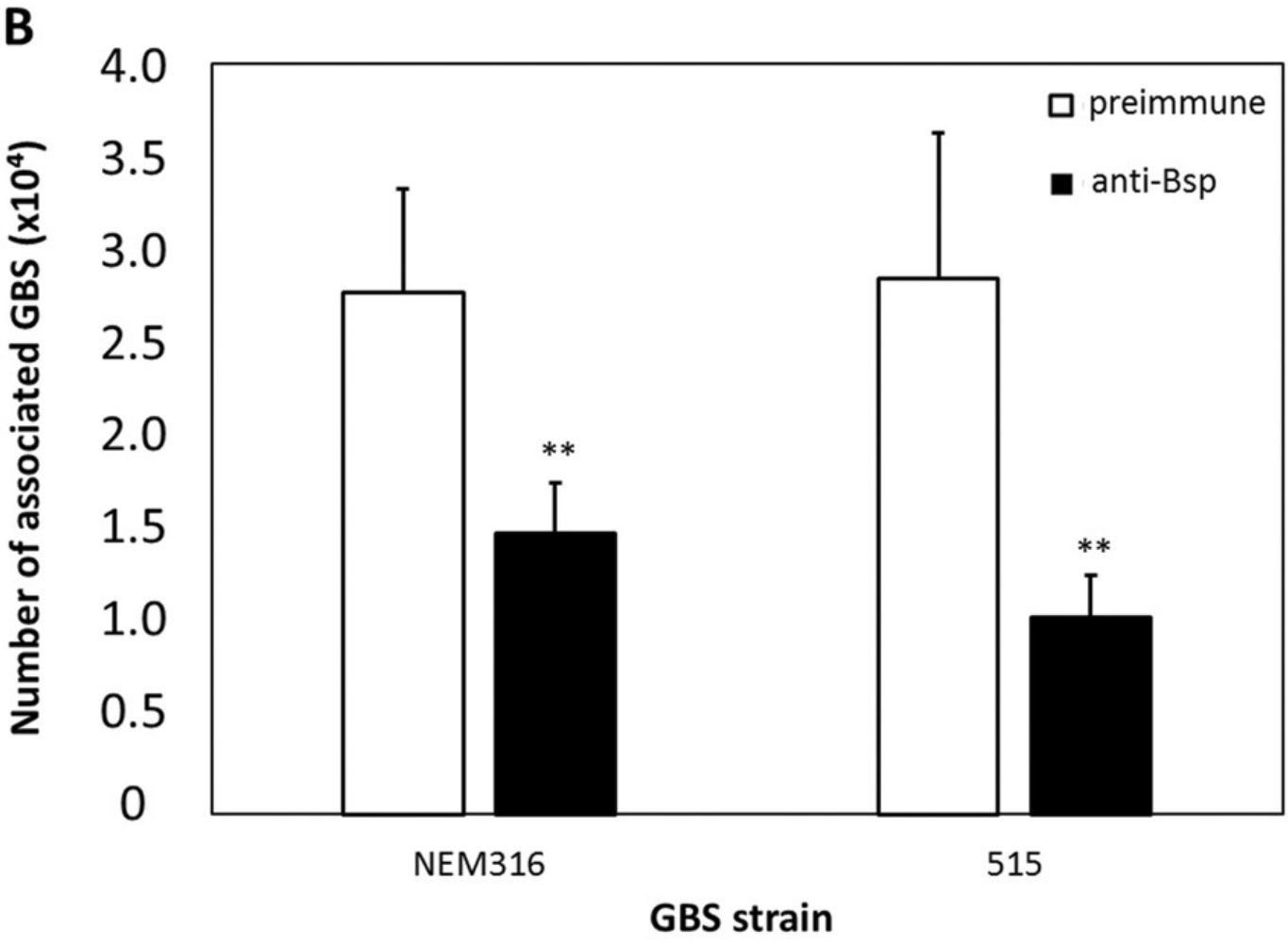

A
Number of associated GBS (x10⁵)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Blank K-SFM
Ca-cell K-SFM
Ca-planktonic K-SFM
NS
NS
NS
NS
NS
NEM316
515
GBS strain

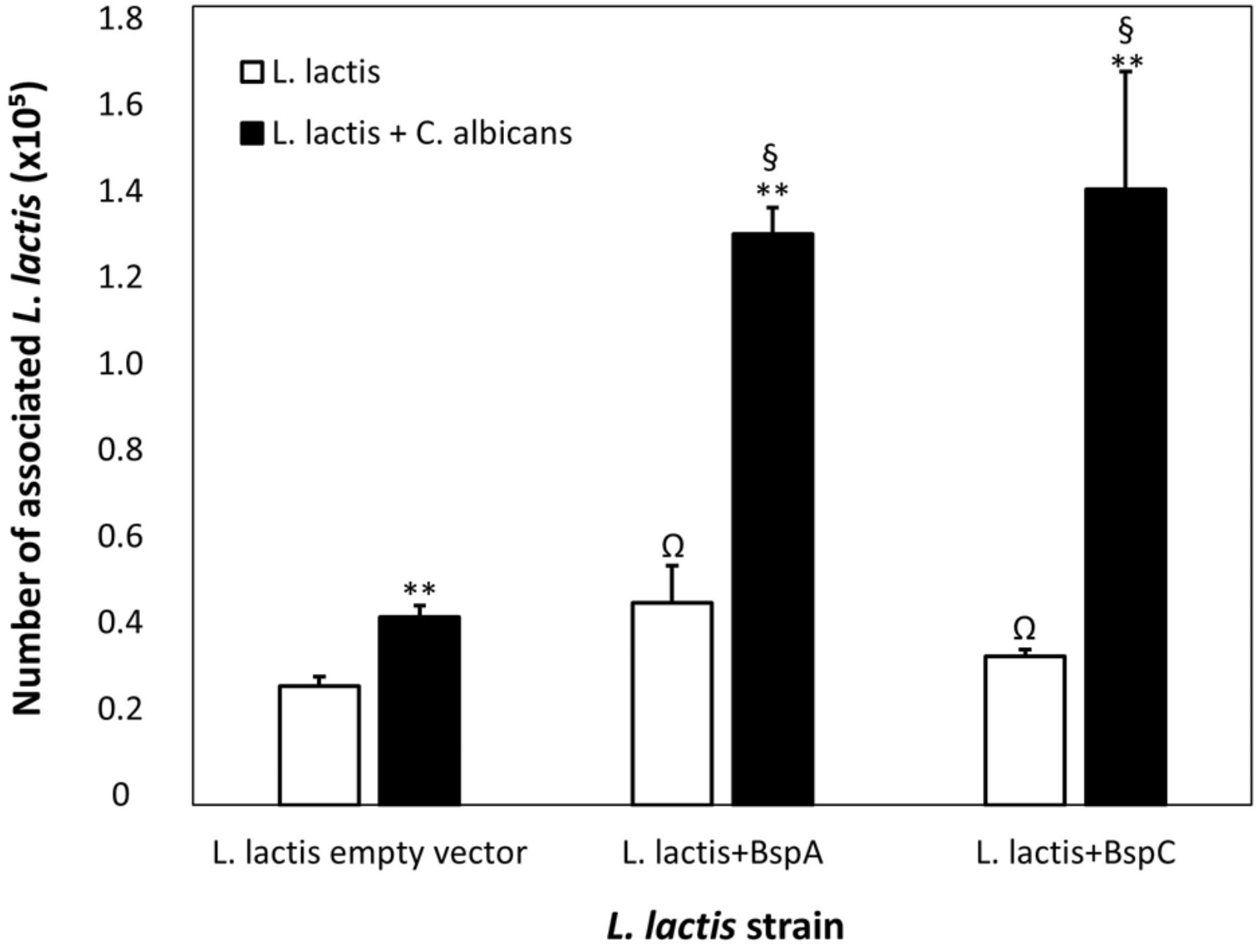

B
Number of associated C. albicans (x10⁶)
9
8
7
6
5
4
3
2
1
0
NS
NS
Ca WT
Ca WT + NEM316
Ca WT + 515

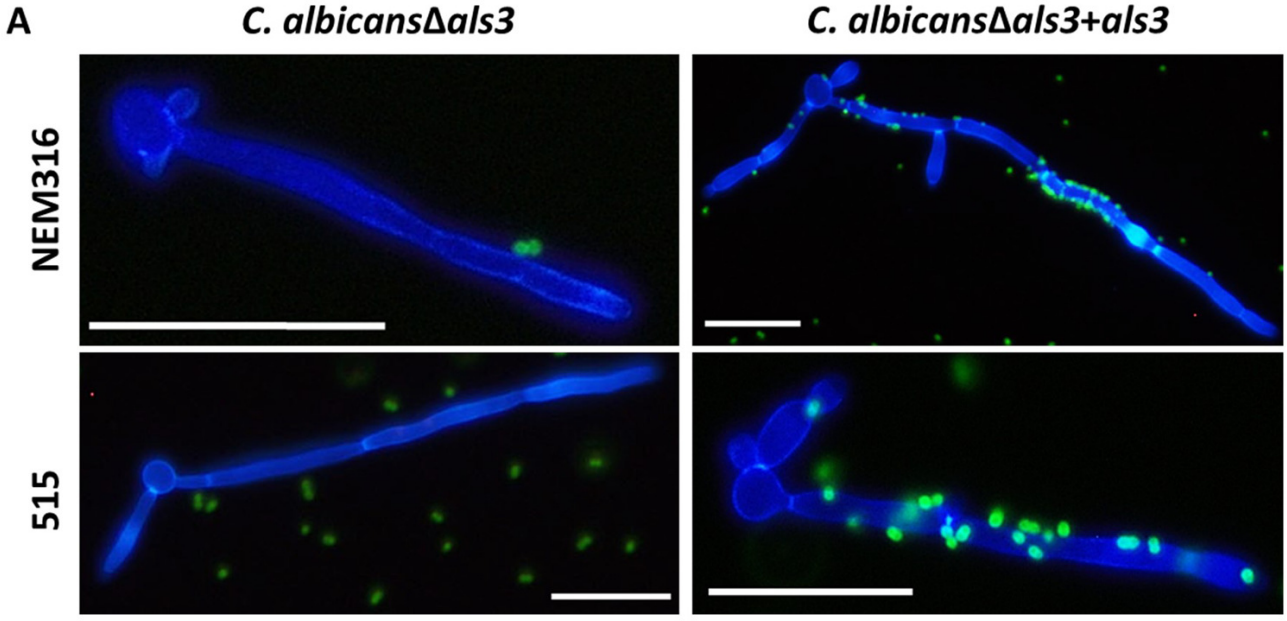

C
Number of associated GBS (x10⁵)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
GBS
GBS + Ca
**
**
NEM316
515
GBS strain

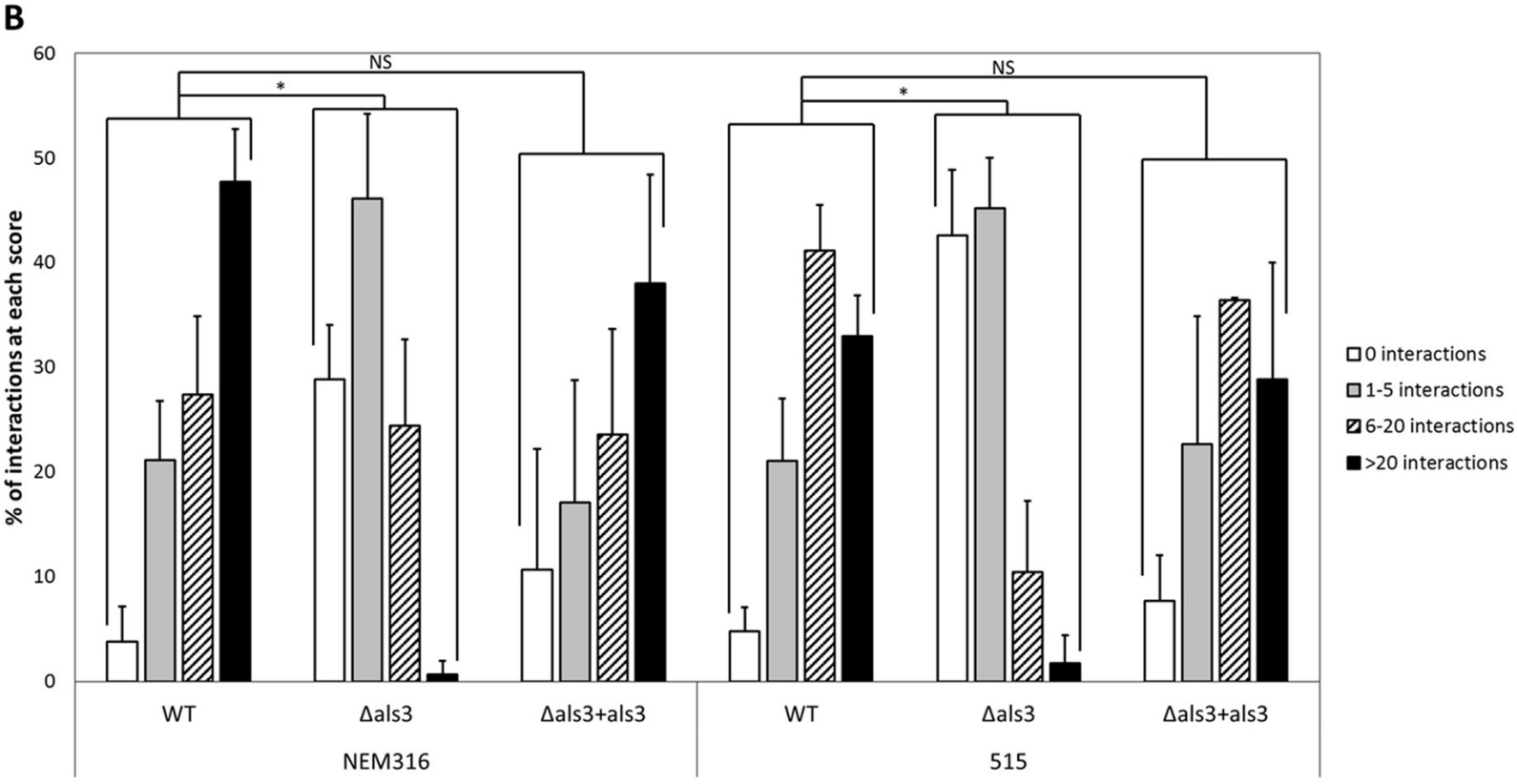

D
Number of associated C. albicans (x10⁵)
10
9
8
7
6
5
4
3
2
1
0
**
**
Ca WT
Ca WT + NEM316
Ca WT + 515

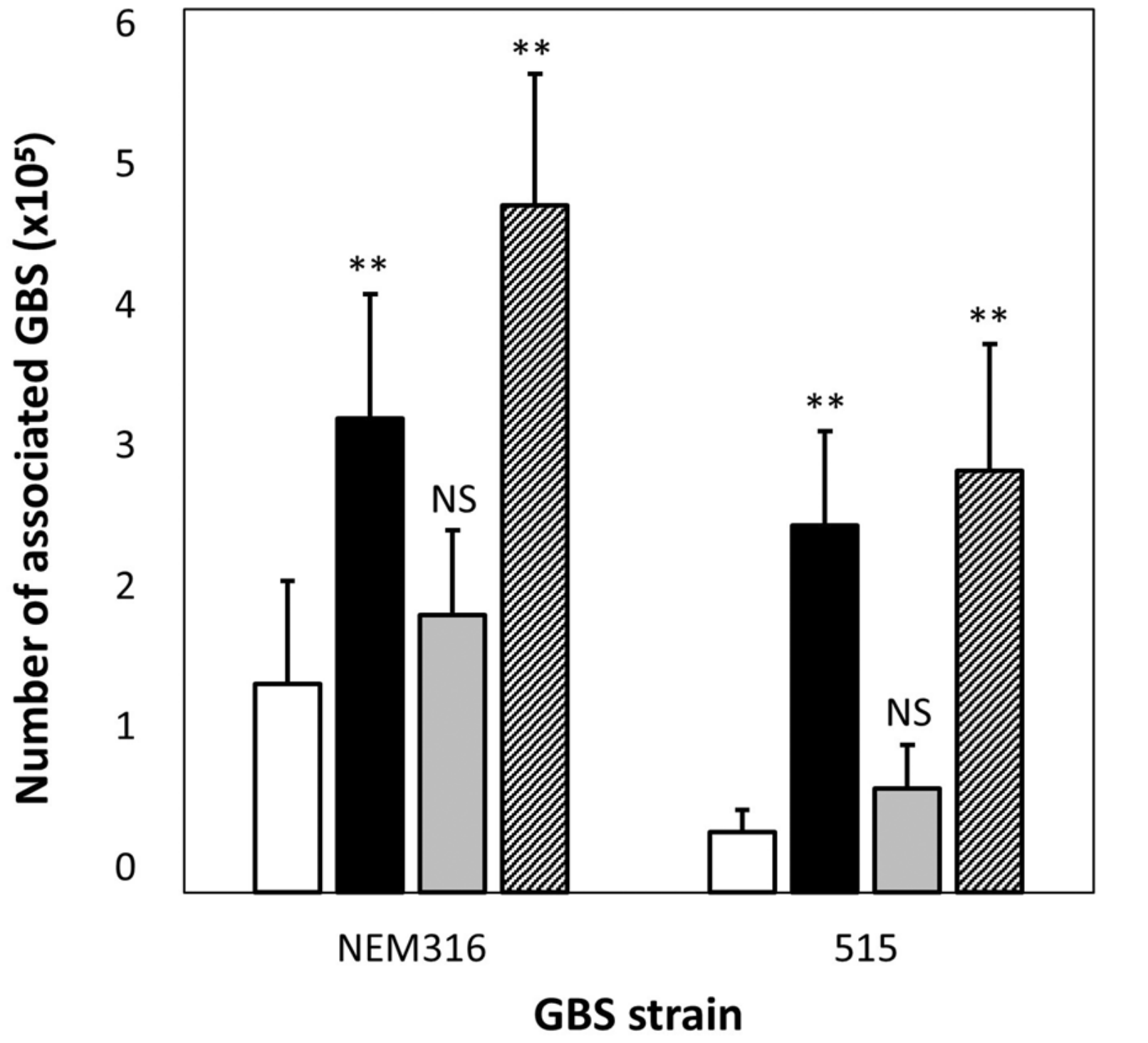

A
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Number of associated GBS (x10⁵)
GBS
GBS+C. albicans
**
§
**
Ω
515 WT
515 ΔbspC
GBS strain

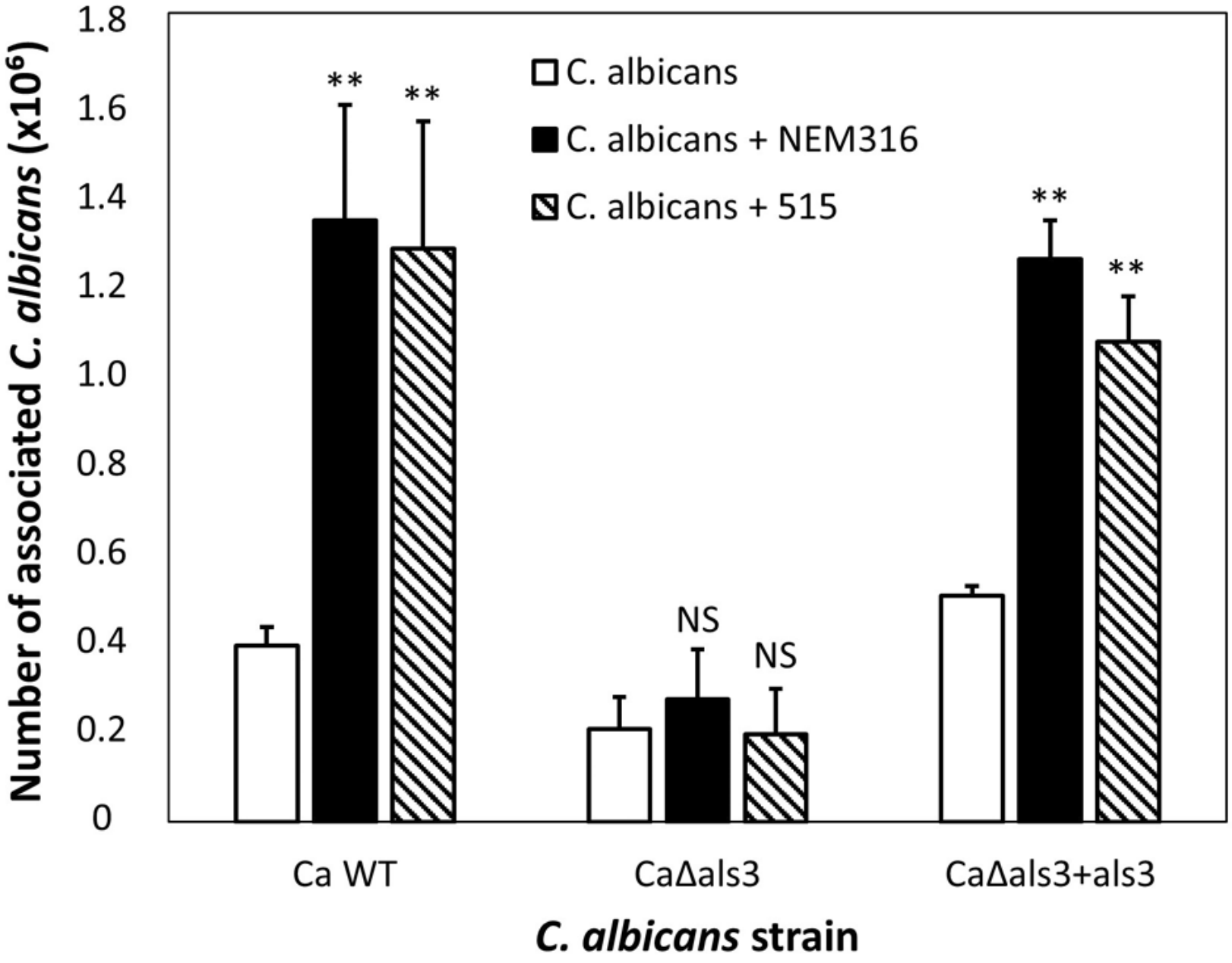

B
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Number of associated GBS (x10⁴)
preimmune
anti-Bsp
**
**
NEM316
515
GBS strain


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Number of associated L. lactis (x10⁵)
L. lactis
L. lactis + C. albicans
**
§
**
Ω
Ω
§
**
L. lactis empty vector
L. lactis+BspA
L. lactis+BspC
L. lactis strain

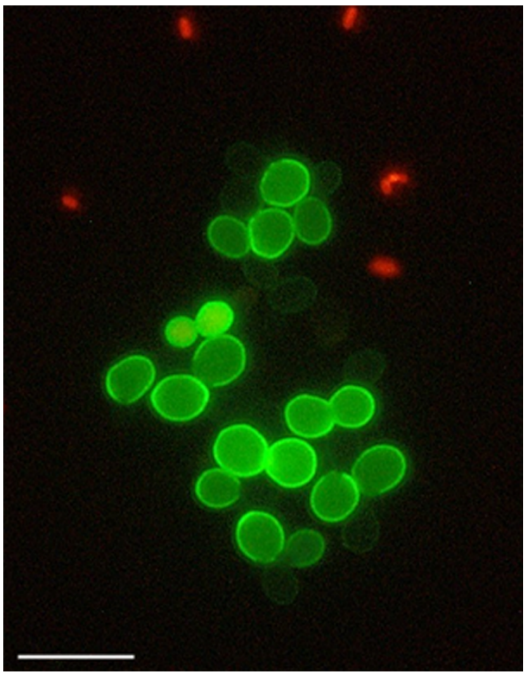

A
C. albicansΔals3
C. albicansΔals3+als3
NEM316
515


B
% of interactions at each score
60
50
40
30
20
10
0
NS
*
NS
*
WT
Δals3
Δals3+als3
WT
Δals3
Δals3+als3
NEM316
515
0 interactions
1-5 interactions
6-20 interactions
>20 interactions

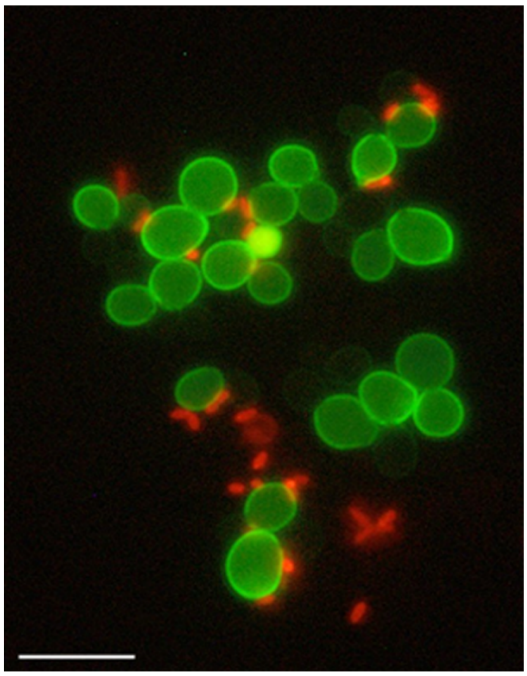

Number of associated GBS (x10⁵)
6
5
4
3
2
1
0
**
**
NS
**
**
NS
NEM316
515
GBS strain
GBS only
GBS + C. albicans WT
GBS + C. albicansΔals3
GBS + C. albicansΔals3+als3


1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Number of associated C. albicans (x10⁶)
C. albicans
C. albicans + NEM316
C. albicans + 515
**
**
NS
NS
**
**
Ca WT
CaΔals3
CaΔals3+als3
C. albicans strain

A) pMSP ctl



B) pMSP-*bspA*



C) pMSP-*bspC*